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# PULLULANASE VARIANTS AND METHODS FOR PREPARING SUCH VARIANTS WITH PREDETERMINED PROPERTIES

#### FIELD OF THE INVENTION

The present invention relates to variants of pullulanases and to methods for constructing such variants with altered properties, including stability (e.g., thermostability), pH dependent activity, substrate specificity, such as increased isoamylase activity, or specific activity; specific activity at a given pH and/or altered substrate specificity, such as an altered pattern of substrate cleavage or an altered pattern of substrate inhibition.

## BACKGROUND OF THE INVENTION

Starches such as corn, potato, wheat, manioc and rice starch are used as the starting material in commercial large-scale production of sugars, such as high fructose syrup, high maltose syrup, maltodextrins, amylose, G4-G6 oligosaccharides and other carbohydrate products such as fat replacers.

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# Degradation of starch

Starch usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages. Amylopectin is partially degraded by alpha-amylase, which hydrolyzes the 1,4-alpha-glucosidic linkages to produce branched and linear oligosaccharides. Prolonged degradation of amylopectin by alpha-amylase results in the formation of so-called alpha-limit dextrins that are not susceptible to further hydrolysis by the alpha-amylase. Branched oligosaccharides can be hydrolyzed into oligosaccharides a debranching by enzyme. The remaining branched oligosaccharides can be depolymerized to D-glucose by glucoamylase, which hydrolyzes linear oligosaccharides into Dglucose.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. Amylose is degraded into shorter linear

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oligosaccharides by alpha-amylase, the linear oligosaccharides being depolymerized into D-glucose by glucoamylase.

In the case of converting starch into a sugar, the starch is depolymerized. The depolymerization process consists of a pretreatment step and two or three consecutive process steps, namely a liquefaction process, a saccharification process and, depending on the desired end product, optionally an isomerization process.

## 10 Pre-treatment of native starch

Native starch consists of microscopic granules that are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation.

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## Liquefaction

During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase (e.g., Termamyl™, available from Novozymes A/S, Denmark). The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The pH generally lies between about 5.5 and 6.2. In order to ensure optimal enzyme stability under these conditions, calcium is added, e.g., 1 mM of calcium (40 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

## Saccharification

After the liquefaction process the maltodextrins are converted into dextrose by addition of a glucoamylase (e.g.  $AMG^{TM}$ , available from Novozymes A/S) and a debranching enzyme, such as an isoamylase (see, e.g., US Patent No. 4,335,208) or a pullulanase (e.g., Promozyme®, available from Novozymes A/S;

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see US Patent No. 4,560,651). Before this step the pH is reduced to a value below 4.5, e.g. about 3.8, maintaining the high temperature (above 95°C) for a period of, e.g., about 30 min. to inactivate the liquefying alpha-amylase to reduce the formation of short oligosaccharides called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

The temperature is then lowered to 60°C, glucoamylase and debranching enzyme are added, and the saccharification process proceeds for about 24-72 hours.

Normally, when denaturing the alpha-amylase after the liquefaction step, a small amount of the product comprises panose precursors, which cannot be degraded by pullulanases or AMG. If active amylase from the liquefaction step is present during saccharification (i.e., no denaturing), this level can be as high as 1-2% or even higher, which is highly undesirable as it lowers the saccharification yield significantly. For this reason, it is also preferred that the alpha-amylase is one which is capable of degrading the starch molecules into long, branched oligosaccharides (such as, e.g., the Fungamyl<sup>TM</sup>-like alpha-amylases) rather than shorter branched oligosaccharides.

# Isomerization

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When the desired final sugar product is, e.g., high fructose syrup, the dextrose syrup may be converted into fructose by enzymatic isomerization. After the saccharification process the pH is increased to a value in the range of 6-8, preferably about pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucose isomerase (such as Sweetzyme<sup>TM</sup>, available from Novozymes A/S).

# Debranching enzymes

Debranching enzymes which can attack amylopectin are divided into two classes: isoamylases (E.C. 3.2.1.68) and pullulanases (E.C. 3.2.1.41), respectively. Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished

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from pullulanases by the inability of isoamylase to attack pullulan, and by their limited action on alpha-limit dextrins.

When an acidic stabilized alpha-amylase is used for the purpose of maintaining the amylase activity during the entire saccharification process (no inactivation), the degradation specificity should be taken into consideration. It is desirable in this regard to maintain the alpha-amylase activity throughout the saccharification process, since this allows a reduction in the amyloglucidase addition, which is economically beneficial and reduces the AMG<sup>TM</sup> condensation product isomaltose, thereby increasing the DE (dextrose equivalent) yield.

It will be apparent from the above discussion that the known starch conversion processes are performed in a series of steps, due to the different requirements of the various enzymes in terms of, e.g., temperature and pH. It would therefore be desirable to be able to engineer one or more of these enzymes, e.g., pullulanases, so that the overall process could be performed in a more economical and efficient manner. One possibility in this regard is to engineer the otherwise thermolabile pullulanases so as to render them more stable at higher temperatures.

# BRIEF DISCLOSURE OF THE INVENTION

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The inventors have modified the amino acid sequence of a pullulanase to obtain variants with improved properties, based on the three-dimensional structure of the pullulanase Promozyme® (available from Novozymes A/S, Denmark). The variants have altered physicochemical properties, e.g., an altered pH optimum, improved thermostability, increased specific activity or an altered cleavage pattern.

Accordingly, the object of the present invention is to provide a method for constructing pullulanases having altered properties, in particular to provide a method for constructing pullulanases having improved thermostability, altered pH dependent activity and/or altered substrate specificity, such as increased isoamylase activity.

Thus, in its broadest aspect, the present invention relates to a method for constructing a variant of a parent

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pullulanase, wherein the variant has at least one altered property as compared to said parent pullulanase, which method comprises:

- i) analyzing the structure of the pullulanase to identify, on the basis of an evaluation of structural considerations, at least one amino acid residue or at least one structural region of the pullulanase, which is of relevance for altering said property;
- ii) constructing a variant of the pullulanase, which as compared to the parent pullulanase, has been modified in the amino acid residue or structural part identified in i) so as to alter said property; and

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iii) testing the resulting pullulanase variant for said property.

The property that may be altered by the above methods of invention may be, e.g., thermostability, pH dependent activity, specific activity, orsubstrate Thus, the variant may have, specificity. e.g., thermostability, higher activity at a lower pH, an altered pH optimum, improved thermostability, or increased specific activity, such as increased isoamylase activity.

Although it has been described in the following that modification of the parent pullulanase in certain regions and/or positions is expected to confer a particular effect to the thus produced pullulanase variant (such as an improved thermostability or an increased isoamylase activity), it should be noted that modification of the parent pullulanase in any of such regions may also give rise to any other of the abovementioned effects. For example, any of the regions and/or positions mentioned as being of particular interest with respect to, e.g., improved thermostability, may also give rise to, e.g., higher activity at a lower pH, an altered pH optimum, or increased specific activity, such as increased isoamylase activity.

Further aspects of the present invention relates to variants of a pullulanase, the DNA encoding such variants and methods of preparing the variants. Still further aspects of the present invention relates to the use of the variants for

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various industrial purposes, in particular for processes where sweeteners are made from starch. Other aspects of the present invention will be apparent from the below description as well as from the appended claims.

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# BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the DNA plasmid pCA36 harboring the gene encoding the pullulanase from *Bacillus deramificans* (SEQ ID NO: .3).

10 APPENDIX 1 shows the structural coordinates for the solved crystal structure of Promozyme®

APPENDIX 2 shows a sequence alignment between Promozyme® (SEQ ID NO: 2), the pullulanase from *Bacillus deramificans* (SEQ ID NO: 4), and the pullulanase from *Bacillus acidopullulyticus* (SEQ ID NO: 6) described in FEMS Mic. Let. (1994) 115, 97-106.

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## DETAILED DISCLOSURE OF THE INVENTION

## Pullulanases

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As explained above, pullulanases are enzymes classified in EC 3.2.1.41 and such enzymes are characterized by their ability to hydrolyze the alpha-1,6-glycosidic bonds in, for example, amylopectin and pullulan.

A particularly interesting pullulanase is the pullulanase from *Bacillus acidopullulyticus* described in US 4,560,651 (hereinafter referred to as Promozyme®). Promozyme® has the amino acid sequence set forth in amino acids 1-921 of SEQ ID NO: 1. The three-dimensional structure of Promozyme® is described below.

Another interesting pullulanase is the pullulanase from Bacillus deramificans described in US 5,736,375. This enzyme has the amino acid sequence set forth in amino acid sequence 1-928 of SEQ ID NO: 3. Homology building of the tree-dimensional structure of the above-mentioned pullulanase is described below.

In general, a preferred pullulanase suitable for the purpose described herein should have one or more of the following properties:

- i) A three-dimensional structure homologous to  $\operatorname{Promozyme}^{\otimes}$ .
- ii) An amino acid sequence which is at least 40% homologous to SEQ ID NO: 1 or SEQ ID NO: 3, preferably at least 50%, e.g., at least 60%, such as a least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homologous to SEQ ID NO: 1 or SEQ ID NO: 3.
  - iii) A nucleic acid sequence which hybridizes to the nucleic acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:3.

The structural homology referred to above in i) above is based on other sequence homologies, hydrophobic cluster analysis or by reverse threading (Huber, T; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998)) and which by any of these methods is predicted to have the same tertiary structure

as Promozyme®, wherein the tertiary structure refers to the overall folding or the folding of Domains N1, N2, A, B, and C. Alternatively, a structural alignment between Promozyme® and homologous sequences may be used to identify equivalent positions.

For example, the homology between various pullulanase with known amino acid sequence has been compiled in the below matrix:

10			1	2	3	4	5	6	7	8	9	10
	1.	pula_kleae	100	86	59	51	52	53	52	52	55	50
	2.	pula_klepn		100	58	51	51	53	53	53	53	52
	3.	w81973			100	55	56	52	55	54	51	56
	4.	r56989				100	98	60	76	54	56	76
15	5.	sp929mat					100	61	78	54	57	78
	6.	fervido_x						100	61	57	54	62
	7.	sp734							100	56	54	91
	8.	r71616								100	54	56
•	9.	w09257									100	54
20	10.	. Promozyme	Ð									100

- 1. Pula\_kleae: Pullulanase from *Klebsiella aerogenes* (*J. Bacteriol*. (1987) 169, 2301-2306).
- 2. Pula\_klepn: Pullulanase from Klebsiella pneumonia (Mol.
- 25 Microbiol. (1990) 4, 73-85; J. Bacteriol. (1985) 164, 639-645;
  J. Bacteriol. (1989) 171, 3673-3679).
  - 3. W81973: Pullulanase fragment from zea mays (WO 98/50562).
  - 4. r56989: Mature pullulanase from *Bacillus deramificans* T 89.117D (EP 0 605 040).
- 30 5. sp929mat: Mature part of pullulanase from Bacillus deramificans (US Patent 5,736,375).
  - 6. fervido\_x: Mature part of pullulanase from Fervidobacterium pennavorans Ven5 (Appl. Environ. Microb. (1997) 63, 1088-1094).
- 35 7. sp734: Mature pullulanase from Bacillus acidopullulyticus (FEMS Mic. Let. (1994) 115, 97-106.
  - 8. r71616: Pullulanase from Thermus sp. (JP 07023783).

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9. w09257: Pullulanase from *Bacillus sp*. KSM-AP 1378 (WO 96/35794).

The above homology calculations were determined by use of the GAP program from the UWGCG package using default values for GAP penalties, i.e., GAP creation penalty of 3.0 and GAP extension penalty of 0.1 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

A sequence alignment between Promozyme® (SEQ ID NO: 1), the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) and the pullulanase from *Bacillus acidopullulyticus* (SEQ ID NO: 5) described in FEMS Mic. Let. (1994) 115, 97-106, is shown in Appendix 2.

# 15 Three-dimensional structure of pullulanase

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Promozyme® was used to elucidate the three-dimensional structure forming the basis for the present invention.

The structure of Promozyme® was solved in accordance with the principle for x-ray crystallographic methods, for example, as given in X-Ray Structure Determination, Stout, G.K. and Jensen, L.H., John Wiley & Sons, Inc. NY, 1989.

The structural coordinates for the solved crystal structure of Promozyme® using the isomorphous replacement method are given in standard PDB format (Protein Data Bank, Brookhaven National Laboratory, Brookhaven, CT) as set forth in Appendix 1. It is to be understood that Appendix 1 forms part of the present application. In the context of Appendix 1, the following abbreviations are used: WAT refers to water or to calcium. Amino acid residues are given in their standard three-letter code.

The structure of said Pullulanase is made up of five globular domains, ordered N1 being 1-310 (which may be subdivided into N1' being residues 1-111, N1''being residues 112-158 and 261-310, and N1''' being residues 159-261), N2, A, B, and C. The domains can be defined as being residues 1-310 for domain N1, 311-420 for Domain N2, residues 421-556 and 596-835 for domain A, residues 557-595 for Domain B, residues 596-922 for Domain C, wherein the numbering refers to the amino

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acid sequence in SEQ ID NO: 1. Features of Domains N1, A, B and C of particular interest are described below.

## Domain N1

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Domain N1 contains in this particular pullulanase an extra loop of 100 residues compared to the pullulanase from Bacillus acidopullulyticus having the amino acid sequence shown in SEQ ID NO: 5. The loop is also present in the pullulanase from Bacillus deramificans having the amino acid sequence shown in SEO ID NO: 3.

Part of the N2 domain is homologeous to the N1 domain of Pseudomonase amyloderamosa isoamylase (1bf2.pdb from Brookhaven database).

#### 15 Domain A

Domain A is the largest domain and contains the active site which comprises a cluster of three amino acid residues, D622, D736 and E651, spatially arranged at the bottom of a cleft in the surface of the enzyme. The structure of Domain A shows an overall fold in common with the alpha-amylases for which the structure is known, viz. the (beta/alpha) 8 barrel with eight central beta strands (numbered 1-8) and eight flanking alpha-helices. The beta-barrel is defined by McGregor, J. Prot. Chem. 7:399, 1988. The C-terminal end of the beta strand 1 is connected to helix 1 by a loop denoted loop 1 and an identical pattern is found for the other loops, although the loops show some variation in size and some can be quite extensive.

The eight central beta-strands in the (beta/alpha) 8 barrel superimpose reasonably well with the known structures of family 13 (Henrissat B. Biochem. J. (1991) 280, 309-316 and Henrissat B. and Bairoch A. Biochem. J. (1993) 293, 781-788). This part of the structure, including the close surroundings of the active site located at the C-terminal end of the beta-strands, shows a high degree of homology with isoamylases.

In contrast, the loops connecting the beta-strands and alpha helices display a high degree of variation from the known structures of family 13 enzymes. These loops constitute the

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structural context of the active site, and the majority of the contacts to the substrate are found among residues located in these loops. Distinguishing characteristics such as substrate specificity, substrate binding, pH activity profile, substrate cleavage pattern, and the like, are determined by specific amino acids and the positions they occupy in these loops.

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#### Domain B

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Domain B, also referred to as loop 3 of the (beta/alpha) 8 barrel, in comprises amino acid residues 557-595 of the amino acid sequence shown in SEQ ID NO: 1. The most striking difference to other family 13 enzymes is the short amino acid sequence. This short sequence loop are of the same size as the isoamylase loop 3 and spatially positioned close to the active site residues and in close contact to the substrate.

#### Domain C

Domain C in Promozyme® comprises amino acid residues 596-922 of the amino acid sequence shown in SEQ ID NO: 1. Domain C is composed entirely of beta-strands which form a single 8-stranded sheet structure that folds back on itself, and thus may be described as a beta-sandwich structure. One part of the beta-sheet forms the interface to Domain A.

# 25 Substrate Binding Site

Parts of the loop discussed above in the context of domains A, B and N2 are of particular interest for substrate interaction and active site reactivity. In particular, in domain A, residues 439-443 in loop 1, residues 490-514 in loop 2, residues 621-628 in loop 4, residues 652-668 in loop 5, residues 679-694 in loop 6, residues 733-740 in loop 7 and residues 787-796 in loop 8; in domain B, residues 553-564 and 581-592 in loop 3; in domain N2, residues 400-404, wherein residue positions correspond to the amino acids in the amino acid sequence in SEQ ID NO: 1.

Homology building of Bacillus d ramificans pullulanase or other pullulanases.

The structure of the *Bacillus deramificans* pullulanase (SEQ ID NO: 3) was model built on the structure disclosed in Appendix 1 herein. The structure of other pullulanases may be built analogously.

A model structure of a pullulanase can be built using the Homology program or a comparable program, e.g., Modeller (both from Molecular Simulations, Inc., San Diego, CA). The principle is to align the sequence of the pullulanase with the known structure with that of the pullulanase for which a model structure is to be constructed. The structurally conserved regions can then be built on the basis of consensus sequences. In areas lacking homology, loop structures can be inserted, or sequences can be deleted with subsequent bonding of the e.g., necessary residues using, the program Subsequent relaxing and optimization of the structure should be done using either Homology or another molecular simulation program, e.g., CHARMm from Molecular Simulations.

# Methods for designing novel pullulanase variants

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In a first aspect the present invention relates to a method for producing a variant of a parent pullulanase, wherein the variant has at least one altered property as compared to the parent pullulanase, the method comprising:

- i) modeling the parent pullulanase on the three-dimensional structure of SEQ ID NO: 1 depicted in Appendix 1 to produce a three-dimensional structure of the parent pullulanase;
- ii) identifying in the three-dimensional structure obtained in step (i) at least one structural part of the parent pullulanase, wherein an alteration in the structural part is predicted to result in an altered property;
- iii) modifying the nucleic acid sequence encoding the parent pullulanase to produce a nucleic acid sequence encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to the structural part; and
- iv) expressing the modified nucleic acid sequence in a host cell to produce the variant pullulanase.

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# Structural part

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The structural parts which is identified in step ii) of the method of the invention may be composed of one amino acid residue. Normally, however, the structural part comprises more than one amino acid residue, typically constituting one of the above-mentioned parts of the pullulanase structure such as one of the N1, N2, A, B, or C domains, an interface between any of these domains, a calcium binding site, a loop structure, the substrate binding site, or the like.

The structural or functional considerations may involve an analysis of the relevant structure or structural part and its contemplated impact on the function of the enzyme. For example, an analysis of the functional differences between pullulanases and the various isoamylases may be used for assigning certain properties of Promozyme® or homologeous model builded structure to certain parts of the Promozyme® or homologeous model build structure or to contemplate such relationship. For instance, differences in the pattern or structure of loops surrounding the active site may result in differences in access to the active site of the substrate and thus differences in substrate specificity and/or cleavage pattern.

Furthermore, parts of a pullulanase involved in substrate binding, and thus, for example, substrate specificity and/or cleavage, thermostability, and the like, have been identified (vide infra).

The modification of an amino acid residue or structural region is typically accomplished by suitable modifications of a nucleic acid sequence encoding the parent enzyme in question. The modification may be substitution, deletion or insertion of an amino acid residue or a structural part.

The property to be modified may be stability (e.g., thermostability), pH dependent activity, substrate specificity, such as decreased condensation reactions, isoamylase like activity etc. Thus, the altered property may be an altered specific activity at a given pH and/or altered substrate specificity, such as an altered pattern of substrate cleavage or an altered pattern of substrate inhibition.

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In step ii) of the method according to the invention the part of the structure to be identified is preferably one which in the folded enzyme is believed to be in contact with the substrate (cf. the disclosure above in the section entitled "Substrate Binding Site") or involved in substrate specificity and/or cleavage pattern, and/or one which is contributing to the pH or temperature profile of the enzyme, or is otherwise responsible for the properties of the pullulanase.

Described in the following are specific types of variants that have been designed by use of the method of the invention.

The variants of the invention may comprise additional modifications in addition to the modifications described herein. The variants preferably have an amino acid sequence having more than 40% homology with SEQ ID NO: 1, SEQ ID NO: 3, or SEQ ID NO: 5, preferably more than 50%, e.g., more than 60%, such as more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98% or more than 99% homology with the amino acid sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or SEQ ID NO: 5.

In the present context the term "homologous to" or "homology" (also sometimes referred to as "similarity") is used in it conventional meaning and the "homology" between two amino acid sequences may be determined by use of any conventional algorithm, preferably by use of the GAP program from the UWGCG package using default values for GAP penalties, i.e. GAP creation penalty of 3.0 and GAP extension penalty of 0.1 (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711). The method is also described in S.B. Needleman and C.D. Wunsch, Journal of Molecular Biology, 48, 443-445 (1970).

# 35 Alternative methods of designing pullulanase variants

The three-dimensional structure of a pullulanase is influenced by the presence (or absence) of the structural spatial location of ions, such as especially calcium ion (also

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sodium ions may be present). Such information may be used as a parameter when designing a pullulanase variant based on informations extracted from one parent pullulanase.

In one embodiment of the invention the parent pullulanase is characterized by having calcium ion(s) in the structure.

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A specific example of such parent pullulanase is Klebsiella sp. mentioned above.

In another embodiment of the invention the parent pullulanase is a pullulanase which do not have metal ion(s) in the structure, in particular calcium(s) ions.

A specific example of a parent pullulanase which do not have metal ion(s) in the structure is PROMOZYME®

In general, informations in respect of structural parts (as defined below) and/or structural elements, e.g., metal ions present in the three-dimensional structure of a parent pullulanase, may be used for as a parameter when model building other corresponding parent pullulanases having, e.g., metal ion(s) in corresponding fixed spatially location(s).

A parent pullulanase may have from no ions to one or more ions in the structure, typically calcium and/or sodium ions. Parent pullulanases having spatially similar located ions (as a solved three-dimensional structure) will also spatially have a similar three dimensional structure. This means that modeling of such a corresponding parent pullulanase with spatially fix ion(s) corresponding to that of, e.g., Promozyme® (disclosed in Appendix 1), said corresponding parent pullulanase having at least 50% homology in the primary structure (as defined above) when compared to SEQ ID NO: 1, 3, or 5, may according to the out as described below. invention be carried corresponding parent pullulanase has at least 60%, or at least 70%, at least 80%, or at least 90% homology in the primary structure when compared to SEQ ID NO: 1, 3, or 5, the modeling will be even more precise.

Thus, in an aspect the invention relates to a method of producing a variant of a parent pullulanase having an altered property relative to the parent pullulanase, wherein the parent pullulanase has the sequence of SEQ ID Nos: 2, 4, 6, or has a

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sequence homology of at least 40% to the sequence of SEQ ID NO: 2, 4, or 6, said method comprising

(a) identifying in the three-dimensional structure depicted in Appendix 1 at least one structural part wherein an alteration in a corresponding structural part in said parent pullulanase is predicted to result in an altered property; wherein the altered property is selected from the group consisting of thermostability, pH dependent activity, substrate specificity, isoamylase like activity, specific activity, altered pattern of substrate cleavage, or an altered pattern of substrate inhibition;

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- (b) modifying the sequence of a nucleic acid encoding the parent pullulanase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and
- (d) expressing the modified nucleic acid in a host cell to produce the variant pullulanase;

wherein the variant has alpha-amylase enzymatic activity and has at least one altered property relative to the parent.

Alternatively, the invention relates to a method of producing a variant of a parent pullulanase having an altered property relative to the parent pullulanase, wherein the parent pullulanase has the sequence of SEQ ID Nos: 2, 4, or 6, or has a sequence homology of at least 40 % to the sequence of SEQ ID NO: 2, 4, or 6, said method comprising

- (al) providing a three-dimensional pullulanase structure that consists of one or more of domains N1 , N2, A, B, and C, in particular N1'';
- (a2), do or do not contain one or more metal ions, in particular calcium or sodium ion(s), in the structure;
- (a3) a three dimensional structure corresponding/equivalent to the three dimensional structure in shown in APPENDIX 1, one or more of the above mentioned domains;
- (b) identifying in the three-dimensional structure at least one structural part of the parent wherein an alteration in said structural part or corresponding structural part in

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said parent pullulanase is predicted to result in said altered property and wherein said altered property is selected from the group consisting of thermostability, pH dependent activity, substrate specificity, isoamylase like activity, specific activity, altered pattern of substrate cleavage, or an altered pattern of substrate inhibition;

- (c) modifying the sequence of a nucleic acid encoding the parent pullulanase to produce a nucleic acid encoding a deletion, insertion, or substitution of one or more amino acids at a position corresponding to said structural part; and
- (d) expressing the modified nucleic acid in a host cell to produce the variant alpha-amylase,

wherein the variant has alpha-amylase enzymatic activity and has at least one altered property relative to the parent.

In an embodiment the parent pullulanase consists of domains N1'', N2, A, B, and C.

In an embodiment the parent pullulanase consists of domains N1', N2, A, B, and C.

In an embodiment the parent pullulanase consists of domains N2, A, B, and C.

In an embodiment the parent pullulanase consists of domains N1', N1'', N2, A, B, and C.

In an embodiment the parent pullulanase consists of domains N1 $^{\prime\prime}$ , N1 $^{\prime\prime}$ , N2, A, B, and C.

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# Properties to be modified

As mentioned above, the property to be modified may be stability (e.g., thermostability), pH dependent activity, substrate specificity, such as increased isoamylase activity, or specific activity. Thus, the altered property may be an altered specific activity at a given pH and/or altered substrate specificity, such as an altered pattern of substrate cleavage or an altered pattern of substrate inhibition.

In a particular interesting embodiment of the invention the property to be modified is the thermostability of the enzyme.

# Common structural elements

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Common structural elements referred to in vi) means elements involved in keeping the three dimensional structure of the enzyme, e.g., metal ions in the structure etc.

# 5 Thermostability

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In the present context, the term "thermostable" "thermostability") refers in general to the fact that the pullulanase variants according to the invention have an improved thermostability compared to the relevant parent pullulanase. The degree of improvement in thermostability can vary according to factors such as the thermostability of the parent pullulanase and the intended use of the pullulanase variant, i.e., whether it is primarily intended to be used for liquefaction or for saccharification or both. It will be apparent from the discussion below that for saccharification, the enzyme variant should maintain a substantial degree of activity during the saccharification step temperature of at least about 63°C, preferably at least about 70°C, while an enzyme variant designed for use liquefaction step should be able to maintain a substantial degree of enzyme activity at a temperature of at least about 95°C.

The improved thermostability of enzyme variants according to the invention can in particular be defined according to one or more of the following criteria:

In one embodiment, the pullulanase variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by differential scanning calorimetry (DSC) using the method described herein.

In another embodiment, the pullulanase variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by an increased half-time  $(T_{1/2})$  of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about

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100%, in the " $T_{1/2}$  assay for liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C. Pullulanase variants according to this definition are suitable for use in the liquefaction step of the starch conversion process.

Alternatively or additionally, a pullulanase variant suitable for use in liquefaction can be defined as having an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after liquefaction" described herein, using a pH of 5.0 and a temperature of 95°C.

In a further embodiment, the enzyme variant of the invention has an improved thermostability (and/or the method of the invention provides a pullulanase with an improved thermostabilty) as defined by an increased half-time  $(T_{1/2})$  of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 25%, most preferably at least about 50%, such as at least about 100%, in the " $T_{1/2}$  assay for saccharification" described herein, using a pH of 4.5 and a temperature of 70°C. Such variants are suitable for use in the saccharification step of the starch conversion process.

Alternatively or additionally, a pullulanase variant suitable for saccharification can be defined as having an improved thermostability as defined by an increased residual enzyme activity of at least about 5%, preferably at least about 10%, more preferably at least about 15%, more preferably at least about 50%, such as at least about 25%, most preferably at least about 50%, such as at least about 100%, in the "assay for residual activity after saccharification" described herein, using a pH of 4.5 and a temperature of 63°C. Preferably, this improved thermostability is also observed when assayed at a temperature of 70°C.

The term "substantially active" as used herein for a given pullulanase variant and a given set of conditions of temperature, pH and time means that the relative enzymatic activity of the enzyme variant is at least about 25%,

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preferably at least about 50%, in particular at least about 60%, especially at least about 70%, such as at least about 90% or 95%, e.g., at least about 99% compared to the relative activity of the parent enzyme tested under the same set of conditions.

One advantage of the thermostable pullulanase of the invention is that they make it possible to perform liquefaction and debranching simultaneously before the saccharification step. This has not previously been possible, since the known pullulanases with acceptable specific activity are thermolabile inactivated at temperatures above 60°C. thermostable pullulanases from Pyrococcus are known, but these have an extremely low specific activity at higher temperatures and are thus unsuitable for purposes of the present invention). By debranching, using the thermostable pullulanases of the invention, during liquefaction together with the action of an alpha-amylase, the formation of panose precursors is reduced, thereby reducing the panose content in the final product and increasing the overall saccharification yield. Ιt is also possible in this manner to extend the liquefaction process time without risking formation of large amount of panose precursors. By prolonging the liquefaction step, the DE yield is increased from 10-15 to, e.g., 15-20, reducing the need for glucoamylase. This reduced glucoamylase requirement is in turn advantageous as the formation of undesired isomaltose is reduced, thereby resulting in an increased glucose yield. In addition, reduced glucoamylase addition enables the saccharification step to be carried out at a higher substrate concentration (higher DS, dry substances, concentration) than the normal approx. 30-35% used according to the prior art. This allows reduced evaporation costs downstream, e.g., in a high fructose corn syrup process, and the saccharification reaction time can also be reduced, thereby increasing production capacity. A further advantage is that alpha-amylase used in the liquefaction process does not need to be inactivated/denatured in this case.

Furthermore, it is also possible to use the thermostable pullulanases of the invention during saccharification, which is advantageous for several reasons. In the conventional starch

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saccharification process, the process temperature is not more than 60°C due to the fact that neither the saccharification pullulanase nor AMG<sup>TM</sup> (i.e., glucoamylase) enzyme allow the sufficiently thermostable to use of temperature. This is a disadvantage, however, as it would be very desirable to run the process at a temperature of above about 60°C, in particular above 63°C, e.g. about 70°C, to reduce microbial growth during the relatively long saccharification step. Furthermore, a higher process temperature normally gives a higher activity per mg of enzyme (higher specific activity), thereby making it possible to reduce the weight amount of enzyme used and/or obtain a higher total enzymatic activity. A higher temperature can also result in a higher dry matter content after saccharification, which would be beneficial in terms of reducing evaporation costs.

In another interesting embodiment of the invention the property to be modified is the substrate specificity of the pullulanase, in particular to modify the substrate specificity of the pullulanase in such a way the variant pullulanase becomes more "isoamylase-like" in the sense of having an increased activity towards high molecular weight branched starchy material such as glycogen and amylopectin. Methods for determining the substrate specificity of pullulanases are discussed in the following section entitled "Methods for determining stability, activity and specificity".

Thus, when used herein, the term "increased isoamylase activity" refers in general to the fact that the pullulanase variants according to the invention exhibits a higher activity towards high molecular weight branched starchy material, such as glycogen and amylopectin as compared to the parent pullulanase.

The increased isoamylase activity of the pullulanase variants according to the invention can in particular be defined according to the below criteria:

In one embodiment the pullulanase variant according to the invention has an increased isoamylase activity as defined by an increase of at least 5%, preferably of at least 10%, more preferably of at least 15%, more preferably of at least 25%,

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most preferably of at least 50%, in particular of at least 75%, such as of at least 100% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10 w/v rabbit liver glycogen solution for a period of 10 min.

In the present context the term "pullulanase activity" is intended to mean that the pullulanase variant in question is capable of degrading pullulan when tested as described in the Examples (see the section entitled "Determination of pullulanase activity).

# Methods for determining stability, activity and specificity

## 15 Thermostability

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# Thermostability Assay 1

Thermostability of pullulanase variants of the invention may be detected by measuring the residual activity by incubating the enzyme under accelerated stress conditions, which comprise: pH 4.5 in a 50 mM sodium acetate buffer without a stabilizing dextrin matrix (such as the approximately 35% dry matter which is normally present during saccharification). The stability can be determined at isotherms of, e.g., 63°C, 70°C, 80°C, 90°C and 95°C, measuring the residual activity of samples taken from a water bath at regular intervals (e.g. every 5 or 10 min.) during a time period of 1 hour. For determining stability for the purpose of liquefaction, a pH of 5.0, a temperature of 95°C and a total assay time of 30 to 120 minutes are used ("assay for activity after liquefaction"). For determining residual stability for the purpose of saccharification, a pH of 4.5, a temperature of 63°C or 70°C and a total assay time of 30 minutes used ("assay for residual activity after are saccharification").

# Thermostability Assay 2 $(T_{1/2})$

Alternatively, the thermostability of pullulanase variants of the invention may be expressed as a "half-time"  $(T_{1/2})$ , which

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is defined as the time, under a given set of conditions, at which the activity of the enzyme being assayed is reduced to 50% of the initial activity at the beginning of the assay. In this case, the " $T_{1/2}$  assay for liquefaction" uses a pH of 5.0 and a temperature of 95°C, while the " $T_{1/2}$  assay for saccharification" uses a pH of 4.5 and a temperature of 70°C. The assay is otherwise performed as described above for the respective assays for residual activity.

#### 10 Activity: Somogyi-Nelson method for determination of reducing sugars

The activity of pullulanases can be measured using the Somogyi-Nelson method for the determination of reducing sugars (J. Biol. Chem. 153, 375 (1944)). This method is based on the principle that sugar reduces cupric ions to cuprous oxide, which reacts with an arsenate molybdate reagent to produce a blue colour that is measured spectrophotometrically. The solution to be measured must contain 50-600 mg of glucose per liter. The procedure for the Somogyi-Nelson method is as follows:

Sample value: Pipet 1 ml of sugar solution into a test tube. Add 1 ml of copper reagent. Stopper the test tube with a glass bead. Place the test tube in a boiling water bath for 20 minutes. Cool the test tube. Add 1 ml of Nelson's color reagent. Shake the test tube without inverting it. Add 10 ml of deionized water. Invert the test tube and shake vigorously. Measure the absorbance at 520 nm, inverting the test tube once immediately prior to transfer of the liquid to the cuvette.

Blank value: Same procedure as for the sample value, but with water instead of sugar solution.

Standard value: Same procedure as for the sample value. Calculations: In the region 0-2 the absorbance is proportional to the amount of sugar.

> 100 (sample - blank) \_\_\_\_\_\_ mg sugar/l =(standard - blank)

(sample - blank) 

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# 100 x (standard - blank)

# Reagents:

1. Somogyi's copper reagent

35.1 g  $Na_2HPO_4.2H_2O$  and 40.0 g potassium sodium tartrate ( $KNaC_4H_4O_2.4H_2O$ ) are dissolved in 700 ml of de-ionized water. 100 ml of 1N sodium hydroxide and 80 ml of 10% cupric sulphate ( $CusO_4.5H_2O$ ) are added. 180 g of anhydrous sodium sulphate are dissolved in the mixture, and the volume is brought to 1 liter with de-ionized water.

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## 2. Nelson's color reagent

50 g of ammonium molybdate are dissolved in 900 ml of deionized water. Then 42 ml of concentrated sulphuric acid are added, followed by 6 g of disodium hydrogen arsenate heptahydrate dissolved in 50 ml of deionized water, and the volume is brought to 1 litre with deionized water. The solution is allowed to stand for 24-48 hours at 37°C before use and is stored in the dark in a brown glass bottle with a glass stopper.

## 20 3. Standard

100 mg of glucose (anhydrous) are dissolved in 1 liter of de-ionized water.

Alternatively, the release of reducing sugars can be measured using a 96 well plate set-up modified after Fox, J.D. & Robyt, J.F. (1991) Anal. Biochem. 195,93-96. Assay conditions are (in brief): 1 ml substrate (e.g. 1% solution) in 50 mM citric acid pH 5 is preincubated at  $60^{\circ}$ C. A zero timepoint is taken 150 micro 1 sample and transferred to a microtiter plate well containing 150 micro 1 solution A + B for reducing sugar determination. The enzymatic reaction is initiated by addition of 100 micro 1 enzyme and time points are taken at T = 1, 2, 3, 4, and 5 min.

After completion of the assay, the plate is developed by incubation at 85  $^{\circ}\text{C}$  for 70 minutes and the plate is read at 540 nm.

Reagents for determination of reducing value: Solution A) and solution B (62 mg copper sulfate pentahydrate and 63 mg L-serine in 50 ml water).

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# Pullulanase specificity

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Methods for the determination and characterization of the profile of action and specificity of pullulanases for various substrates (e.g., amylopectin, glycogen and pullulan) are described by Kainuma et al. in Carbohydrate Research, 61 345-357 (1978). Using these methods, the relative activity of a pullulanase can be determined, and the relative activity of a pullulanase variant according to the invention compared to the relative activity of the parent pullulanase can be assessed, for example to determine whether a pullulanase variant has the desired increased specificity toward high molecular weight saccharides, such as amylopectin, compared to the parent pullulanase.

In order to determine whether the pulluanase variant possesses an increased isoamylase activity as compared to the parent pullulanse the following test may be performed ("assay for isoamylase-like activity"):

1000 mg rabbit liver glycogen is dissolved in 40 ml water to which 0.2% NaOH has been added. 800 mg NaBH4 is added carefully under stirring. The solution is stirred for 48 hours at 25°C after which the reaction is stopped by addition of Amberlite IR-118H (a cation exchanger which removes the boron ions and hence stops the reaction). The solution is filtered to remove the matrix and evaporated to give 10 ml. The solution is then dialyzed extensively against de-ionized water to remove residual boron ions. The parent pullulanase and the pulluanase variant are assayed according to the method of Somogyi-Nelson, using 50 mM sodium acetate, pH values of 4.5, 5.0 or 5.5 and a temperature of 60°C, with a reaction time of 10 minutes. Glucose is used as a standard, a standard curve being made from solutions containing of 0-200 mg glucose/liter.

Clearly, the higher the number of reducing ends formed during the incubation period, the higher "isoamylase activity". The increase in the pullulanase variant's isoamylase activity is expressed as a percentage value based on the original "isoamylase activity" of the parent pullulanase.

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# Pullulanas variants with altered stability

A variant with improved stability (typically increased thermostability) may be obtained by substitution with proline, substitution of histidine with another amino acid, introduction of a disulfide bond, removal of a deamidation site, altering a hydrogen bond contact, filling in an internal structural cavity with one or more amino acids with bulkier side groups, introduction of interdomain interactions, altering distribution, helix capping, or introduction of a salt bridge.

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# Increased mobility regions:

The following regions have an increased mobility in the crystal structure of Promozyme®, and it is presently believed that these regions can be responsible for stability or activity of the enzyme. Improvements of the enzyme can be obtained by mutation in the below regions and positions. Introducing e.g. larger residues or residues having more atoms in the side chain could increase the stability, or, e.g., introduction of residues having fewer atoms in the side chain could be important for the mobility and thus the activity profile of the enzyme. The regions can be found by analysing the B-factors taken from the coordinate file in Appendix 1, and/or from molecular dynamics calculations of the isotropic fluctuations. These can be obtained by using the program CHARMm from MSI (Molecular simulations inc.).

Thus, in order to stabilize mobile regions a preferred variant of a parent pullulanase structure, comprises a modification, e.g., a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1: 408-429 (i.e., 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428 and 429),

300-314 (i.e., 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313 and 314), 35 157-165 (i.e., 157, 158, 159, 160, 161, 162, 163, 164 and 165),

95-113 (i.e., 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105,

106, 107, 108, 109, 110, 111, 112 and 113),

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130-140 (i.e., 130, 131, 132, 133, 134, 135, 136, 137, 138, 139
    and 140),
    232-238 (i.e., 232, 233, 234, 235,236, 237 and 238),
    266-272 (i.e., 266, 267, 268, 269, 270, 271 and 272),
    302-308 (i.e., 302, 303, 304, 305, 306, 307 and 308),
    418-428 (i.e., 418, 419, 420, 421, 422, 423, 424, 425, 426, 427
    and 428),
    500-507 (i.e., 500, 501, 502, 503, 504, 505, 506 and 507),
    659-665 (i.e., 659, 660, 661, 662, 663, 664 and 665) and
    751-755 (i.e., 751, 752, 753, 754 and 755).
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         Similar modifications, e.g.,
                                          substitutions,
    introduced in equivalent positions of other pullulanases.
    Variants of particular interest have a combination of one or
    more of the above with any of the other modifications disclosed
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   herein.
                        other
                                 preferred
       For
             example,
                                            modifications,
    substitutions, which are believed to stabilized mobile regions
    in the structure of the pullulanase from Bacillus deramificans,
    correspond to one or more of the following residues of the
    amino acid sequence set forth in SEQ ID NO: 3:
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    406-427 (i.e., 406, 407, 408, 409, 410, 411, 412, 413, 414,
    415, 416, 417, 418, 419,
    420, 421, 422, 423, 424, 425, 426 and 427),
    298-312 (i.e., 298, 299, 300, 301, 302, 303, 304, 305, 306,
   307, 308, 309, 310, 311 and 312),
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    153-161 (i.e., 153, 154, 155, 156, 157, 158, 159, 160 and 161),
    91-109 (i.e., 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101,
    102, 103, 104, 105, 106, 107, 108 and 109),
    126-136 (i.e., 126, 127, 128, 129, 130, 131, 132, 133, 134, 135
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   and 136),
    230-236 (i.e., 230, 231, 232, 233, 234, 235 and 236),
    264-270 (i.e., 264, 265, 266, 267, 268, 269 and 270),
    300-306 (i.e., 300, 301, 302, 303, 304, 305 and 306),
    416-426 (i.e., 416, 417, 418, 419, 420, 421, 422, 423, 424, 425
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   and 426),
    498-505 (498, 499, 500, 501, 502, 503, 504 and 505),
    656-662 (i.e., 656, 657, 658, 659, 660, 661 and 662) and
    749-753 (i.e., 749, 750, 751, 752 and 753).
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Contemplated combinations of substitutions include according to the invention one or more of: deletion (1-111); deletion(1-113); D562P; G292P; G794P; D148P; N119P; N400S; N400L; N400F; N446S; N446L; N446F; N504S; N504L; N504F; N735S; N735L; N735F; N789S; N789L; N789F; I566A; Q485H; V551I; S492F; D444R; D444K; deletion (154-273).

Multi substitutions of the invention include: deletion(1-111)+D562P; deletion(1-111)+G292P; deletion(1-111)+G794P; deletion(1-111)+N119P+N400S/L/F;deletion(1-111)+D148P; 10 deletion(1-111)+N446L/F; deletion(1-111)+N504S/L/F; deletion(1-111)+N735S/L/F; deletion(1-111)+N789S/L/F; deletion(1-111)+I566A; deletion(1-111)+Q485H; deletion(1-111)+V551I; deletion(1-111)+S492F; eletion(1-111)+D444R/K; eletion(1-111) + deletion (154-273); deletion (1-113) + D562P; deletion (1-111)+G292P; eletion(1-113)+G794P; deletion(1-113)+D148P; 15 eletion(1-113)+N504S/L/F; deletion(1-113)+N735S/L/F; eletion(1-113)+N735S/L/F; 113)+N789S/L/F; deletion(1-113)+I566A; deletion(1-113)+Q485H; deletion(1-113)+V551I; deletion(1-113)+S492F; eletion(1-113) +D444R/K; deletion(1-113) +deletion(154-273); D562P+G292P; 20 D562P+G794P; D562P+D148P; D562P+N119P; D562P+N400S/L/F; D562P+N504S/L/F; D562P+N735S/L/F; D562P+N446S/L/F; D562P+N789S/L/F; D562P+I566A; D562P+Q485H; D562P+V551I; D562P+S492F; D562P+D444R/K; D562P+deletion (154-273); 25 G292P+G794P; G292P+D148P; G292P+N119P; G292P+N400S/L/F; G292P+N504S/L/F; G292P+N446S/L/F; G292P+N735S/L/F; G292P+N789S/L/F; G292P+I566A; G292P+Q485H; G292P+V551I; G292P+S492F; G292P+D444R/K; G292P+deletion (154-273); G794P+D148P; G794P+N119P; G794P+N400S/L/F; G794P+N446S/L/F; 30 G794P+N504S/L/F; G794P+N735S/L/F; G794P +N789S/L/F; G794P+I566A; G794P+Q485H; G794P+V551I; G794P+S492F; G794P+deletion (154-273); 148P+N119P; G794P+D444R/K; D148P+N446S/L/F; D148P+N504S/L/F; D148P+N400S/L/F; D148P+N735S/L/F; D148P+N789S/L/F; D148P+I566A; D148P+Q485H; 35 D148P+V551I; D148P+S492F; D148P+D444R/K; D148P+deletion (154-

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273); N119P+N400S/L/F; N119P+N446S/L/F; N119P+N504S/L/F; N119P+N735S/L/F; N119P+N789S/L/F; N119P+I566A; N119P+Q485H; N119P+V551I; N119P+S492F; N119P+D444R/K; N119P+deletion (154-N400S/L/F+N446S/L/F; N400S/L/F+N504S/L/F; 273); 5 N400S/L/F+N735S/L/F; N400S/L/F+N789S/L/F; N400S/L/F+I566A; N400S/L/F+S492F; N400S/L/F+Q485H; N400S/L/F+V551I; N400S/L/F+D444R/K; N400S/L/F+deletion (154-273);N446S/L/F+N504S/L/F; N446S/L/F+N735S/L/F; N446S/L/F+N789S/L/F; N446S/L/F+I566A; N446S/L/F+Q485H; N446S/L/F+V551I; N446S/L/F+S492F; N446S/L/F+D444R/K; N446S/L/F+deletion (154-10 N504S/L/F+N735S/L/F; N504S/L/F+N789S/L/F;273); N504S/L/F+V551I; N504S/L/F+Q485H; N504S/L/F+I566A; N504S/L/F+S492F; N504S/L/F+D444R/K; N504S/L/F+deletion (154-273); N735S/L/F+N789S/L/F; N735S/L/F+I566A; N735S/L/F+Q485H; N735S/L/F+S492F; N735S/L/F+D444R/K; N735S/L/F+V551I; 15 N735S/L/F+deletion (154-273); N789S/L/F+I566A; N789S/L/F+Q485H; N789S/L/F+V551I; N789S/L/F+S492F; N789S/L/F+D444R/K; N789S/L/F+deletion (154-273);I566A+Q485H; I566A+V551I; I566A+S492F; I566A+D444R/K; I566A+deletion (154-273);Q485H+V551I; Q485H+S492F; Q485H+D444R/K; Q485H+deletion 20 (154-273); V551I+S492F; V551I+D444R/K; V551I+deletion (154-273); S492F+D444R/K; S492F+deletion (154-273); D444R/K+deletion (154-273); deletion (1-111)+D562P+N400S/L/F; deletion(1-111)+G292P+ N400S/L/F; deletion(1-111)+G794P+N400S/L/F; deletion(1deletion(1-111) + N119P + N400S/L/F;111) +D148P+N400S/L/F; 25 deletion(1-111)+N446L/F+N400S/L/F; deletion(1-111)+N504S/L/F+deletion(1-111) +N735S/L/F+N400S/L/F; deletion(1-N400S/L/F; deletion(1-111)+I566A+N400S/L/F; 111) +N789S/L/F+N400S/L/F; deletion(1-111)+V551I+ deletion(1-111)+Q485H+N400S/L/F;N400S/L/F; deletion(1-111)+S492F+N400S/L/F; deletion(1-30 111) +D444R/K+N400S/L/F; deletion(1-111) +deletion(154-273) + N400S/L/F; deletion(1-111)+D562P; deletion(1-111)+ deletion(1-111)+D148P;deletion(1eletion(1-111)+G794P; 111) +N119P+N400S/L/F; deletion(1-111) +N446L/F; deletion(1deletion(1-35 111) +N504S/L/F; deletion(1-111) +N735S/L/F;

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111) +N789S/L/F; deletion(1-111)+I566A; deletion(1-111)+Q485H; deletion(1-111)+V551I;

Furthermore, it is envisaged from the structure that deletion of certain amino acid residues will confer increased stability, such as increased thermostability, to the thus produced variant. Variants, which are believed to be of particular importance, comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Deletion of the peptide fragment 158-275, such as a deletion starting from position 158, 159, 160 or 161 and ending at position 270, 271, 272, 273, 274 or 275, i.e., the longest deletion will be deletion of the peptide fragment 158-275 and the shortest deletion will be deletion of the peptide fragment 151-270.

Other deletions which are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

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Deletion of the peptide fragment 1-315, such as deletion of the peptide fragment 1-314, 1-313, 1-312, 1-311, 1-310, 1-309, 1-308, 1-307, 1-306, 1-305, or 1-304.

Furthermore, the following deletions are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Deletion of the peptide fragment 1-115, such as deletion of the peptide fragment 1-114, 1-113, 1-112, 1-111, 1-110, 1-109, 1-108, 1-107, 1-106 or 1-105.

Similar deletions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

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For example, it is envisaged that deletion of the below amino acid residues will confer increased stability, such as increased thermostability, to the thus produced variant of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3):

Deletion of the peptide fragment 154-273, such as a deletion starting from position 154, 155, 156 or 157 and ending at position 268, 269, 270, 271, 272 or 273, i.e. the longest deletion will be deletion of the peptide fragment 154-273 and the shortest deletion will be deletion of the peptide fragment 157-268.

Other deletions which are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 3:

Deletion of the peptide fragment 1-313, such as deletion of the peptide fragment 1-312, 1-311, 1-310, 1-309, 1-308, 1-307, 1-306, 1-305, 1-304, or 1-303.

Furthermore, the following deletions are expected to confer increased stability, such as increased thermostability, to the pullulanase variant comprises a deletion of amino acid residues corresponding to the following residues of the amino acid sequence set forth in SEQ ID NO: 3:

Deletion of the peptide fragment 1-111, such as deletion of the peptide fragment 1-113, 1-111, 1-110, 1-109, 1-108, 1-107, 1-106, 1-105, 1-104, 1-103, 1-102 or 1-101.

#### Cavities and crevices

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The structure of the pullulanase contains a number of unique internal cavities, which may contain water, and a number of crevices. In order to increase the stability, preferably the thermostability, of the pullulanase it may be desirable to reduce the number or size of cavities and crevices, e.g., by introducing one or more hydrophobic contacts, preferably achieved by introducing amino acids with bulkier side chains in the vicinity or surroundings of the cavity or crevice. For instance, the amino acid residues to be modified are those that are involved in the formation of a cavity or crevice.

In order to determine which amino acid residues of a given enzyme are involved in the formation of cavities or crevices the Conolly program is normally used (B. Lee and F.M. Richards, J. Mol. Biol. 55, 379-400 (1971)). The program uses a probe with a certain radius to search the external and internal surface of the protein. The smallest crevice observable in this way has the probe radius.

To analyze the solved structure of Promozyme®, a modified version of the Connolly program included in the program of INSIGHT was used. In the first step, the water molecules and the ions were removed by unmerging these atoms from the solved structure. By using the command MOLECULE SURFACE SOLVENT the solvent accessible surface area was calculated for all atoms and residues using a probe radius of 1.4 Å, and displayed graphically together with the model of the solved structure. The internal cavities are then seen as dot surfaces with no connections to the external surface.

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Suggestions for specific modifications to fill out the cavities are given below. By using the homology built structures and/or comparisons based on sequence alignment, mutations for homologous structures of pullulanases can be made.

Accordingly, in a further aspect the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

- a) identifying an internal cavity or crevice in the three-dimensional structure of the parent pullulanase;
- b) substituting at least one amino acid residue involved in the formation of a cavity or crevice with another amino acid residue which increases the hydrophobic interaction and/or fills out or reduces the size of the cavity or crevice;
  - c) optionally repeating steps a) and b) recursively;
- d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);
  - e) preparing the variant resulting from steps a) d);
- f) testing the stability and/or the temperature dependent activity profile of the variant; and

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- g) optionally repeating steps a) f) recursively; and
- h) selecting a variant having increased stability and/or an altered temperature dependent activity profile as compared to the parent pullulanase.

In a preferred embodiment of the invention the variant pullanase provided by the above method have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

It will be understood that the cavity or crevice is identified by the amino acid residues surrounding said cavity or crevice, and that modification of said amino acid residues are of importance for filling or reducing the size of the cavity crevice. Preferably, the modification orsubstitution with a bulkier amino acid residue, i.e. one with a greater side chain volume or with an increased number of atoms in the side chain. For example, all the amino acids are bulkier than Gly, whereas Tyr and Trp are bulkier than Phe. particular amino acid residues referred to below are those that in a crystal structure have been found to flank the cavity or crevice in question.

In a preferred embodiment, the variant of a pullulanase, in order to fill, either completely or partly, cavities or crevices located internally or externally in the structure, comprises a modification, e.g., a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1: 406, 394, 568, 573 576, 563, 557, 396, 392, 515, 583, 442, 792, 767, 732, 760, 783, 740, 688, 478, 534, 550, 627, 314.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

35 G406A, P394F/W/I/L, I568L/F, Y573W, T576N/L/I, S563T, T557N, A396V/L/I, V392, N515M/L/I, V583I/F/L, D442Q, S792Y/F, V767Q/E/L/I, V732I/L, D760Q/E/F/Y, L783F/Y, L740Q,

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D688Y/F/E/Q/R/K, L478Q/R, L534F/Y/I, M550F/Y/I/L, L627F/Y/I, L314I.

Similar modifications, e.g., substitutions, may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, the variant of a pullulanase may also comprise one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEO ID NO: 3:

566, 485, 487, 437, 775, 779, 551, 428, 492, 495, 392, 621, 437+503, 674+664 and 823.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

I566A, Q485H, M487L, D437H, Q775H, E779D, V551I, I428Y/F, S492F, V495I/F/Y, P392Y, L621Q, D437H+D503Y, V674+L664F and L823V.

#### Disulfide bonds

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A variant with improved stability (typically improved thermostability) as compared to the parent pullulanase may be obtained by introducing new interdomain and intradomain contacts, such as establishing inter- or intradomain disulfide bridges.

Accordingly, a further aspect of the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

- a) identifying in the three-dimensional structure of the parent pullulanase two or more amino acid residues which, when substituted with cysteines, are capable of forming a disulfide bond;
- b) substituting the amino acids identified in a) with cysteines;
  - c) optionally repeating steps a) and b) recursively;

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d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);

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e) preparing the variant resulting from steps a) - d);

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- f) testing the stability of said variant; and
- g) optionally repeating steps a) f) recursively; and
- h) selecting a variant having increased stability as compared to the parent pullulanase.

In a preferred embodiment of the invention the variant pullanase provided by the above method have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

In order to determine, in the three-dimensional structure of the parent pullulanase, the amino acid residues which, when substituted with cysteines, are capable of forming a disulfide bond, residues with CB atoms less than 4Å from each other, and where the direction of the CA-CB from each residue is pointing towards the other residue are identified. Following the abovementioned guidelines, the below amino acid residues were identified in the amino acid sequence of SEQ ID NO: 1, and it is contemplated that these residues are suitable for cystein replacement, thereby opening up the possibility of establishing one or more disulfide bridges in the variant pullulanase:

K758C+I914C, T916C+A765C, I897C+S819C, P525C+E499C, H286C+T148C.

Similar substitutions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, it is contemplated that the following residues, identified in the amino acid sequence of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3), are suitable for cystein replacement, thereby opening up the possibility of establishing one or more disulfide bridges in the variant pullulanase:

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K756C/I912C, M914C/A763C, V895C/G817C, A523C/E497C, H284C/T144C.

# Surface charge distribution

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A variant with improved stability (typically improved thermostability) as compared to the parent pullulanase may be obtained by changing the surface charge distribution of the pullulanase. For example, when the pH is lowered to about 5 or below histidine residues typically become positively charged and, consequently, unfavorable electrostatic interactions on the protein surface may occur. By engineering the surface charge of the pullulanase one may avoid such unfavorable electrostatic interactions that in turn leads to a higher stability of the pullulanase.

Therefore, a further aspect of the present invention relates to method for constructing a variant of a parent pullulanase, the method comprising:

- a) identifying, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His;
- b) substituting, on the surface of the parent pullulanase, at least one amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His with an uncharged amino acid residue;
  - c) optionally repeating steps a) and b) recursively;
- d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);
  - e) preparing the variant resulting from steps a) d);
  - f) testing the stability of said variant; and
  - g) optionally repeating steps a) f) recursively; and
- h) selecting a variant having increased stability as compared to the parent pullulanase.

As will be understood by the skilled person it may also, in some cases, be advantageous to substitute an uncharged amino acid residue with an amino acid residue bearing a charge or, alternatively, it may in some cases be advantageous to substitute an amino acid residue bearing a charge with an amino

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acid residue bearing a charge of opposite sign. Thus, the above-mentioned method may easily be employed by the skilled person also for these purposes. In the case of substituting an uncharged amino acid residue with an amino acid residue bearing a charge the above-mentioned method may be employed the only difference being steps a) and b) which will then read:

- a) identifying, on the surface of the parent pullulanase,
   at least one uncharged amino acid residue;
- b) substituting, on the surface of the parent pullulanase, at least one uncharged amino acid residue with a charged amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His.

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Also in the case of changing the sign of an amino acid residue present on the surface of the pullulanase the above method may be employed. Again, compared to the above method, the only difference being steps a) and b) which, in this case, read:

- a) identifying, on the surface of the parent pullulanase, at least one charged amino acid residue selected from the group consisting of Asp, Glu, Arg, Lys and His;
- b) substituting, on the surface of the parent pullulanase, at least one charged amino acid residue identified in step a) with an amino acid residue having an opposite charge.

Thus, Asp may be substituted with Arg, Lys or His; Glu may be substituted with Arg, Lys or His; Arg may be substituted with Asp or Glu; Lys may be substituted with Asp or Glu; and His may be substituted with Asp or Glu.

In a preferred embodiment of the invention the variant pullulanase provided by the above method(s) have increased thermostability as compared to the parent pullulanase. The thermostability of a given variant may be assessed as described in the above section entitled "Methods for determining stability, activity and specificity".

In order to determine the amino acid residues of a pullulanase, which are present on the surface of the enzyme, the surface accessible area are measured using the DSSP program (Kabsch and Sander, *Biopolymers* (1983), 22, 2577-2637). All

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residues having a surface accessibilty higher than 0 is regarded a surface residue.

The amino acid residues found on the surface of  $Promozyme^{\oplus}$  using the above method are as follows:

5 E526, Q544, E760, N338, N228, N181,

and it is contemplated that the following substitutions are of particular interest:

E526H, Q544E, E760Q, N338K/R, N228DE/, N181K/R.

Similar substitutions may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

For example, the variant of a pullulanase may also comprise one or more modifications, e.g., substitutions, corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3: 444, 530, 710 and 855.

In a more preferred embodiment, the variant of a pullulanse comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 3:

D444R/K, K530Y/F/L, N710R and T855K.

#### Other modifications

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Variants with improved stability, in particular variants with improved thermostability, can be obtained by improving existing or introducing new interdomain or intradomain contacts. Such improved stability can be achieved by the modifications listed below.

Thus, one preferred embodiment of the invention relates to a variant of a parent pullulanase which has an improved stability and one or more salt bridges as compared to the parent pullulanase, wherein said variant comprises a modifications, e.g., a substitution, in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

301, 385, 298, 299, 385 and 299+385, in particular L301R, N385R, H298R, N299R, N385D and N299R+N385D.

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Similar modifications, e.g., substitutions, may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

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For example, it is contemplated that the following substitutions in the pullunanase having the amino acid sequence set forth in SEQ ID NO: 3 will enhance the stability of the enzyme: T891D, S892K, T891D+S892K and N400R.

In another preferred embodiment, the variant of the pullulanase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 1:

G293P, K151P, K122P, N315P, N374P, N793P, A446P, G672P, G668P, T556P

In a further interesting embodiment of the invention, the variant of the pullulanase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 3:

D562P, G794P, G292P, D148P, N119P, D314P, N373P, N792P, G671P, G667P, and T554P.

Analogously, it may be preferred that one or more histidine residue(s) present in the parent pullulanase is (are) substituted with a non-histidine residues such as Y, V I, L, F, M, E, Q, N, or D. Accordingly, in another preferred embodiment, the variant of the parent pullulanase comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 3: H422Y/F/L, H483Y/F/L, H543Y/F/L/N and H613Y/F/L.

It may be preferred that one or more asparagine or glutamine residues present in the parent pullulanase is or are substituted with a residue lacking the amide group on the side chain. Preferably, such asparagines or glutamine residues are substituted with S, T, V, L and/or F amino acid residues. Accordingly, in another preferred embodiment, the variant of the parent pullulanase comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or

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more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

Q543, Q339, N337, Q380, Q353, N384, N286, N298, N227, Q227, Q210, N180, Q259, N583, N790, N793, N505, N788, N736, N684, N681, preferably Q543S/T/V/L/F, Q339S/T/V/L/F, N689 or N337S/T/V/L/F, Q380S/T/V/L/F, Q353S/T/V/L/F, N384S/T/V/L/F, N286S/T/V/L/F, N298S/T/V/L/F, N227S/T/V/L/F, Q227S/T/V/L/F, Q210S/T/V/L/F, N180S/T/V/L/F, Q259S/T/V/L/F, N583S/T/V/L/F, N790S/T/V/L/F, N793S/T/V/L/F, N505S/T/V/L/F, N788S/T/V/L/F,

The corresponding residues found in the pullulanase from Bacillus deramificans (SEQ ID NO: 3) include:

N736S/T/V/L/F, N684S/T/V/L/F, N689S/T/V/L/F and N681S/T/V/L/F.

and N789, preferably N400, N504, N717, N735 N446. N446S/T/V/L/F, N504S/T/V/L/F, N717S/T/V/L/F, N400S/T/V/L/F, N735S/T/V/L/F, and N789S/T/V/L/F.

Moreover, it is contemplated that modifications, e.g. substitutions, in the region linking the N2 and the A domain, as well as other regions linking other domains, will confer additional stability, such as an increased thermostability, to Thus, in an interesting embodiment of the the enzyme. invention, the pullulanase variant comprises one or modifications, e.g., substitutions, in the domain-linking regions (e.g., the region linking the N2 and A domains).

Examples of such modifications include one or more of the following substitutions in the pullulanase from Bacillus 25 deramificans (SEQ ID NO: 3):

111, 112,

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158-160 (i.e., 158, 159 and 160),

270-274 (i.e., 270, 271, 272, 273 and 274),

302-314 (i.e., 302, 303, 304, 305, 306, 307, 308, 309, 310, 30 311, 312, 313 and 314) and

408-426 (i.e., 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425 and 426).

Examples of specific substitutions are: S111T/V/L, N112S/T/Q, S158Y/F/T, L159Y/K/R/A/S/T, G160A/S/T, D270E/S/T, 35 L271V/I, V272I, T273N/D/E/Y/F, V274I, N302V/L/Y, N305V/L/Y, S306T/V, Q308K/R/A/S/T, Y309F, Y310E/D/Q/N/L/V/I, D314A/S/T, L409N, D408S/T, A410S/T, D413R/K/S/T, A415S/T, G416S/T/V,

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N418A/V/S/T, S419D/N/T, K421E/Q/S/T/V/A, H422D/L/Y/F, I423L/V/S/T/N/Q, T424S/A and K426A/S/T.

Other substitutions that are considered of particular importance in SEQ ID NO: 3 include D437N and D440N.

Similar modifications, e.g., substitutions, may be introduced in equivalent positions of other pullulanases. Modifications of particular interest are any combination of one or more of the above with any of the other modifications disclosed herein.

Before actually constructing a pullulanase variant to achieve any of the above objectives, it may be convenient to evaluate whether or not the contemplated amino acid modification can be accommodated into pullulanase structure, e.g., in a model of the three-dimensional structure of the parent pullulanase.

# Pullulanase variants with altered substrate specificity

One aim of the present invention is to change the degradation characteristics of a pullulanase. Thus, as Promozyme® (and pullulanases in general) exhibits a low activity towards high molecular weight branched starchy material, such as glycogen and amylopectin, it may be desirable to change this cleavage pattern, e.g., so as to obtain a higher activity against such substrates, in particular when the pullulanase is to be added during the liquefaction process.

An altered substrate specificity may be achieved by modifying the substrate binding area in a parent pullulanse.

Accordingly, the present invention also relates to a method for constructing a variant of a parent pullulanase, the method comprising:

- a) identifying the substrate binding area in a model of the three-dimensional structure of the parent pullulanase;
- b) modifying the substrate binding area by an amino acid substitution, deletion and/or insertion;
  - c) optionally repeating step b) recursively;
- d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

- e) preparing the variant resulting from steps a) d);
- f) testing the substrate specificity of the variant;
- g) optionally repeating steps a) f) recursively; and
- h) selecting a variant having an altered substrate specificity as compared to the parent pullulanase.

The substrate binding area may easily be identified by homology to other family 13 members. The active site residues are identified by homology. The substrate-binding site is identified by the concave cavity containing the active site residues. A substrate model is docked into the cavity. A suitable substrate model is the substrate structure found in the pdb file 1BAG termed GLC. This model can be "docked" into the Promozyme X-ray structure or a modeled Pullulanase 3D structure by superimposing the active site residues in the two structures. In 1BAG one of the active site residues has been mutated into a Gln instead of the native Glu. The active site residues to be superimposed are: D269, Q208 and D176 (1BAG) with D736, E651 and D622 (Promozyme®). The superposition can be made using the program INSIGHTII.

Without being limited to any theory, it is presently believed that binding between a substrate and an enzyme is supported by favorable interactions found within a sphere 10 Å from the substrate molecule, in particular within a sphere of 6 Å from the substrate molecule. Examples of such favorable bonds are hydrogen bonds, strong electrostatic interaction and/or hydrophobic interactions. The following residues of Promozyme® (SEQ ID NO: 1), are within a distance of 10 Å from the "docked" substrate and thus believed to be involved in interactions with said substrate:

30 437, 439, 487, 489, 490, 514, 679, 681, 684, 685, 731, 775, 786,

494-496 (i.e., 494, 495 and 496),

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505-511 (i.e., 505, 506, 507, 508, 509, 510 and 511),

551-559 (i.e., 551, 552, 553, 554, 555, 556, 557, 558 and 559),

35 584-590 (i.e., 584, 585, 586, 587, 588, 589 and 590),

620-626 (i.e., 620, 621, 622, 623, 624, 625, 626),

650-658 (i.e., 659, 651, 652, 653, 654, 655, 656, 657 and 658),

665-668 (i.e., 666, 667 and 668),

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690-693 (i.e., 690, 691, 692 and 693),

734-738 (i.e., 734, 735, 736, 737 and 738) and

789-795 (i.e., 789, 790, 791, 792, 793, 794 and 795).

The following residues of Promozyme® are within a distance of 6 Å from the substrate and thus believed to be involved in interactions with said substrate:

489, 551, 553, 555, 556, 620, 651, 691, 692, 791, 793, 794,

506-510 (i.e., 507, 508, 509 and 510),

586-588 (i.e., 586, 587 and 588),

10 622-624 (i.e., 622, 623 and 624),

653-656 (i.e., 653, 654, 655 and 656) and

735-737 (i.e., 735, 736 and 737),

In a preferred embodiment of the invention, the parent pullulanase is modified in such a way that the variant pulluanase exhibits an increased isoamylase activity compared to the parent pullulanase.

When used herein, the term "increased isoamylase activity" refers in general to the fact that the pullulanase variants according to the invention exhibits a higher activity towards high molecular weight branched starchy material, such as glycogen and amylopectin as compared to the parent pullulanase, cf. above.

In an interesting embodiment of the invention the pullulanase variant has an increased isoamylase activity as defined by an increase of at least 5%, preferably of at least 10%, more preferably of at least 15%, more preferably of at least 25%, most preferably of at least 50%, in particular of at least 75%, such as of at least 100% in the number of reducing ends formed in the "assay for isoamylase-like activity" described herein, using 50 mM sodium acetate, a pH of 4.5, 5.0 or 5.5, a temperature of 60°C and when incubated with a 10 w/v rabbit liver glycogen solution for a period of 10 min.

Similar modifications may be introduced in equivalent positions of other pullulanases. Substitutions of particular interest are any combination of one or both of the above with any of the other modifications disclosed herein.

For example, the following residues of the pullulanase from Bacillus deramificans (SEQ ID NO: 3) are within a distance

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of 10 Å from the "docked" substrate and thus believed to be involved in interactions with said substrate:

435, 437, 485, 487, 488, 512, 677, 679, 682, 683, 729, 773, 784,

5 492-494 (i.e., 492, 493 and 494),

503-509 (i.e., 503, 504, 505, 506, 507, 508 and 509),

549-557 (i.e., 549, 550, 551, 552, 553, 554, 555, 556 and 557),

582-588 (i.e., 582, 583, 584, 585, 586, 587 and 588),

618-624 (i.e., 618, 619, 620, 621, 622, 623 and 624),

10 648-656 (i.e., 648, 649, 650, 651, 652, 653, 654, 655 and 656),

663-666 (i.e., 663, 664, 665 and 666),

688-691 (i.e., 688, 689, 690 and 691),

732-736 (i.e., 732, 733, 734, 735 and 736) and

787-793 (i.e., 787, 788, 879, 790, 791, 792 and 793).

The following residues of the pullulanase from *Bacillus deramificans* (SEQ ID NO: 3) are within a distance of 6 Å from the substrate and thus believed to be involved in interactions with said substrate:

487, 549, 551, 553, 554, 618, 649, 689, 690, 789, 791, 792,

20 504-508 (i.e., 504, 505, 506, 507 and 508),

584-586 (i.e., 584, 585 and 586),

620-622 (i.e., 620, 621 and 622),

651-654 (i.e., 651, 652, 653 and 654) and

733-735 (i.e., 733, 734 and 735).

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Examples of specific modifications in the above-mentioned regions of Bacillus deramificans are: L621I/V, D508M/N/L/T/V, T586I/L/V, T677W/F/Y, Y729F/I/L, D679G/A/V, S732V/T/L/I, N735G/L/V/I/S/T/A and  $\Delta$ (688-691).

#### 30 Pullulanase variants with altered pH dependent activity profile

The pH dependent activity profile can be changed by changing the pKa of residues within 15 Å, in particular by changing the pKa of residues within 10 Å, from the active site residues of the parent pullulanase. Changing the pKa of the active site residues is achieved, e.g., by changing the electrostatic interaction or hydrophobic interaction between functional groups of amino acid side chains of a given amino acid residue and its close surroundings. To obtain a higher

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activity at a higher pH, negatively charged residues are placed near a hydrogen donor acid, whereas positively charged residues placed near a nucleophilic acid will result in higher activity at low pH. Also, a decrease in the pKa can be obtained by reducing the accessibility of water or increasing hydrophobicity of the environment.

It is preferred that the variant in question exhibits a pH optimum which is at least about 0.5 pH units higher or lower, preferably at least about 1.0 pH units higher or lower, than the corresponding pH optimum of the parent pullulanase when tested on a suitable substrate (e.g. pullulan, amylopectin or glycogen).

Furthermore, it is particular preferred that the variant in question exhibits an increased activity in the pH range of from 4 to 5.5 as compared to the parent pullulanase when tested on a suitable substrate (e.g., pullulan, amylopectin or glycogen).

Thus, another aspect of the present invention relates to a method for constructing a variant of a parent pullulanase, the method comprising:

- a) identifying an amino acid residue which is within 15 Å, in particular within 10 Å, from an active site residue of the parent pullulanase in the three-dimensional structure of said parent pullulanse, and which is involved in electrostatic or hydrophobic interactions with an active site residue;
- b) substituting said amino acid residue with another amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue, and which can be accommodated in the structure;
  - c) optionally repeating steps a) and b) recursively;
- d) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b);
  - e) preparing the variant resulting from steps a) d);
  - f) testing the pH dependent activity of said variant; and
  - g) optionally repeating steps a) f) recursively; and
- h) selecting a variant having an altered pH dependent activity as compared to the parent amylase.

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In general, an amino acid residue which is within 15 Å or 10Å, respectively, from an active site residue of the parent pullulanase may be identified by using the INSIGHTII program.

In a preferred embodiment, the variant of a parent pullulanase having an altered pH dependent activity profile as compared to the parent pullulanase comprises a modification, e.g. a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1 (all within 15Å from the active site residues D736, E651, D622):

430, 433, 518, 521, 565, 599, 600, 610, 611, 635, 636, 639, 717, 760, 763, 764, 767, 817,

435-443 (i.e., 435, 436, 437, 438, 439, 440, 441, 442, and 443),

15 486-496 (i.e., 486, 487, 488, 489, 490, 491, 492, 493, 494, 495 and 496),

505-515 (i.e., 505, 506, 507, 508, 509, 510, 511, 512, 513, 514 and 515),

548-560 (i.e., 548, 549, 550, 551, 552, 553, 554, 555, 556,

20 557, 558, 559 and 560),

573-575, (i.e., 573, 574 and 575),

583-595 (i.e., 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594 and 594),

602-604 (i.e., 602, 603 and 604),

25 606-608 (i.e., 606-607 and 608),

616-633 (i.e., 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, and 633),

646-672 (i.e., 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666,

30 667, 668, 669, 670, 671 and 672),

674-696 (i.e., 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695 and 696), 720-722 (i.e. 720, 721 and 722),

725-747 (i.e., 725, 726, 727, 728, 729, 730, 731, 732, 733,

35 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746 and 747), 773-781 (i.e. 773, 774, 775, 776, 777, 778, 779, 780 and 781), 783-797 (i.e. 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796 and 797) and

799-802 (i.e., 799, 800, 801 and 802).

Within 10Å from the active site residues D736, E651, D622:

437, 442, 492, 514, 575, 594, 603, 632, 635, 684, 688, 691,

5 692, 721, 727, 729, 742, 743, 775, 777, 778, 780, 784, 786, 800,

487-490 (i.e., 487, 488, 489 and 490),

507-511 (i.e., 507, 508, 509, 510 and 511),

550-557 (i.e., 550, 551, 552, 553, 554, 555, 556 and 556),

10 585-588 (i.e., 585, 586, 587 and 588),

590-592 (i.e., 590, 591 and 592),

619-628 (i.e., 619, 620, 621, 622, 623, 624, 625, 626, 627 and 628), 648-655 (i.e., 648, 649, 650, 651, 652, 653, 654 and 655),

15 665-671 (i.e., 665, 666, 667, 668, 669, 670 and 671),

676-681 (i.e., 676, 677, 678, 679, 680 and 681),

731-740 (i.e., 731, 732, 733, 734, 735, 736, 737, 738, 739 and 740) and

788-793 (i.e., 788, 789, 790, 791, 792 and 793).

20 Similar modifications may be introduced in equivalent positions of other pullulanases. Variants of particular interest have a combination of one or more of the above with any of the other modifications disclosed herein.

Thus, in another preferred embodiment, the variant of a parent pullulanase having an altered pH dependent activity profile as compared to the parent pullulanase comprises a modification, e.g., a substitution, of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 3 (all within 15 Å from the active site residues D734, E649 and D620):

428, 431, 516, 519, 563, 597, 598, 608, 609, 633, 634, 637, 715, 758, 761, 762, 765, 815,

433-441 (i.e., 433, 434, 435, 436, 437, 438, 439, 440 and 441),

484-494 (i.e., 484, 485, 486, 487, 488, 489, 490, 491, 492, 493

35 and 494),

503-513 (i.e., 503, 504, 505, 506, 507, 508, 509, 510, 511, 512 and 513),

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546-558 (546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557 and 558), 571-573 (i.e., 571, 572 and 573), 581-593 (i.e., 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592 and 593), 600-602 (i.e., 600, 601 and 602), 604-606 (i.e., 604, 605 and 606), 614-631 (i.e., 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630 and 631), 10 644-670 (i.e., 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 665, 666, 667, 668, 669 and 670), 672-694 (i.e., 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690,691, 692, 693 and 694), 15 718-720 (i.e., 718, 719 and 720), 723-745 (i.e., 723, 734, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744 and 745), 771-779 (i.e., 771, 772, 773, 774, 775, 776, 777, 778 and 779), 20 781-795 (i.e. 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794 and 795) and 797-800 (i.e., 797, 798, 799 and 800). Within 10 Å from the active site residues D734, E649 and 25 D620: 435, 440, 490, 512, 573, 601, 605, 630, 669, 682, 686, 689, 690, 719, 725, 727, 740, 741, 773, 775, 776, 778, 782, 784, 798, 485-488 (i.e., 485, 486, 487 and 488), 505-509 (i.e., 505, 506, 507, 508 and 509), 548-555 (i.e., 548, 549, 550, 551, 552, 553, 554 and 555), 583-586 (i.e., 583, 584, 585 and 586), 588-590 (i.e., 588, 589 and 590),

and 626). 646-653 (i.e., 646, 647, 648, 649, 650, 651, 652 and 653), 663-667 (i.e., 663, 664, 665, 666 and 667), 674-679 (i.e., 674, 675, 676, 677, 678 and 679),

617-626 (i.e., 616, 617, 618, 619, 620, 621, 622, 623, 624, 625

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729-738 (i.e., 729, 730, 731, 732, 733, 734, 735, 736, 737 and 738) and

786-791 (i.e., 786, 787, 788, 789, 790 and 791).

Specific examples of substitutions in the above-mentioned positions include D437L/I/V/F, D440L/I/V/F, M486K, M487K, D503L/I/V/F, D508N/L/T/V, T586V/I, M630H and D437L/I/V/F+D440L/I/V/F+D503L/I/V/F.

#### Nomenclature for amino acid modifications

The nomenclature used herein for defining modifications is essentially as described in WO 92/05249. Thus, G406A indicates a substitution of the amino acid G (Gly) in position 406 with the amino acid A (Ala). G406 indicates a substitution of the amino acid G (Gly) with any other amino acid. P394F/W/I/L or P394F,W,I,L indicates a substitution of P394 with F, W, I or L.  $\Delta$ (688-691) indicates a deletion of amino acids in positions 688-691. 412-A-413 indicates an insertion of A between amino acids 412 and 413.

A deletion of Alanine in position A30 is shown as:

20 A30\*

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and insertion of an additional amino acid residue, such as Lysine, is shown as:

A30AK

Where a specific pullulanase contains a "deletion" in comparison with other pullulanases and an insertion is made in such a position this is indicated as:

\*36D

for insertion of an Aspartic acid in position 36.

Multiple mutations are separated by plus signs, i.e.:

A30N+E34S or A30N/E34S

representing mutations in positions 30 and 34 substituting Alanine and Glutamic acid for Asparagine and Serine, respectively.

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine

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in position 30 is mentioned, but not specified, it is to be understood that the Alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

Further, "A30X" means any one of the following substitutions:

A30R, A30N, A30D, A30C, A30Q, A30E, A30G, A30H, A30I, A30L, A30K, A30M, A30F, A30P, A30S, A30T, A30W, A30Y, or A30 V; or in short: A30R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

When used herein, the term "modification" (of a particular amino acid residue) is intended to cover substitution and deletion (of the particular amino acid residue) as well as insertion of one or more amino acid residues after the particular amino acid residue.

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# Polypeptide sequence homology

For purposes of the present invention, the degree of homology may be suitably determined according to the method described in S.B. Needleman and C.D. Wunsch, Journal of Molecular Biology, 48, 443-45, with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The determination may be done by means of a computer program known such as GAP provided in the UWGCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

#### Hybridization

Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al. Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., Cold Spring Harbor, 1989) for 10 min, and prehybridization of the filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 micro g/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same

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solution containing a random-primed (A. P. Feinberg B. and Vogelstein, Anal. Biochem. 132, 6-13 (1983)), <sup>32</sup>P-dCTP-labeled (specific activity > 1 x 10<sup>9</sup> cpm/micro g) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

# Methods of preparing pullulanase variants according to the invention

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# Cloning a DNA sequence encoding a pullulanase

The DNA sequence encoding a parent pullulanase may be isolated from any cell or microorganism producing the pullulanase in question, using various methods well known in the art.

a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the pullulanase to be studied. Then, if sequence of pullulanase amino acid the is the homologous, labelled oligonucleotide probes may be synthesised and used to identify pullulanase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known pullulanase gene could be used as a probe to identify pullulanase-encoding clones, using hybridization and washing conditions of lower stringency.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers, *Tetrahedron Letters*, 22, 1859-1869 (1981) or the method described by Matthes et al. *The EMBO*, 3, 801-805 (1984). In the phosphoroamidite method, oligonucleotides are syn-

thesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

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Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin, wherein the fragments correspond to various parts of the entire DNA sequence, in accordance with techniques well known in the art. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. Science, 239, 487-491(1988).

# Site-directed Mutagenesis

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pullulanase-encoding DNA sequence been has and desirable sites for modification identified, be introduced modifications may using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired modification sites; nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the pullulanase-encoding sequence, is created in a vector carrying the pullulanase gene. Then the synthetic nucleotide, bearing the desired modification, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. Biotechnology 2, 639-646 (1984). 4,760,025 disclose the introduction of oligonucleotides encoding multiple modifications by performing minor alterations cassette. However, an even greater variety modifications can be introduced at any one time by the Morinaga method because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing modifications into a pullulanase-encoding DNA sequences is described in Nelson and Long Analytical Biochemistry, 180, 147-151 (1989). It involves a 3-step generation of a PCR fragment containing the desired modification introduced by using a chemically synthesized DNA

strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the modification may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

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#### Random Mutagenesis

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Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent pullulanase may be conveniently performed by use of any method known in the art.

In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent pullulanase, wherein the variant exhibits an altered property, such as increased thermostability, increased stability at low pH and at low calcium concentration, relative to the parent pullulanase, the method comprising:

- (a) subjecting a DNA sequence encoding the parent pullulanase to random mutagenesis,
  - (b) expressing the mutated DNA sequence obtained in step(a) in a host cell, and
- (c) screening for host cells expressing a pullulanase variant which has an altered property relative to the parent pullulanase.

Step (a) of the above method of the invention is preferably performed using doped primers.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) ir-

radiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane (EMS), sodium bisulphite, formic acid, sulphonate and When nucleotide analogues. such agents are used, the mutagenesis is typically performed by incubating the sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions that are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the pullulaase enzyme by any published technique, using, e.g., PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

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Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and modification in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% modifications in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, inter alia, ensures that introduction of stop codons is avoided (L.J. Jensen et al. Nucleic Acid Research, 26, 697-702 (1998).

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent pullulanase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, 1, 1989, pp. 11-15).

A mutator strain of *E. coli* (Fowler et al., *Molec. Gen. Genet.*, 133, 1974, 179-191), *S. cereviseae* or any other

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microbial organism may be used for the random mutagenesis of the DNA encoding the pullulanase by, e.g., transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent pullulanase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harbored in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as Bacillus subtilis, licheniformis, Bacillus lentus, Bacillus brevis, Bacillus Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis,

Streptomyces lividans or Streptomyces murinus; and gram negative bacteria such as E. coli.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

# Localized random mutagenesis

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The random mutagenesis may be advantageously localized to a part of the parent pullulanase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

# 25 General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

- Select regions of interest for modification in the
   parent enzyme
  - 2. Decide on mutation sites and non-mutated sites in the selected region
  - 3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed
    - 4. Select structurally reasonable mutations
  - 5. Adjust the residues selected by step 3 with regard to step 4.

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6. Analyze by use of a suitable dope algorithm the nucleotide distribution.

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- 7. If necessary, adjust the wanted residues to genetic code realism, e.g. taking into account constraints resulting from the genetic code, e.g. in order to avoid introduction of stop codons; the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted
  - 8. Make primers

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9. Perform random mutagenesis by use of the primers 10.Select resulting pullulanase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One such algorithm is described by Tomandl, D. et al., 1997, Journal of Computer-Aided Molecular Design 11:29-38. Another algorithm is DOPE (Jensen, LJ, Andersen, KV, Svendsen, A, and Kretzschmar, T (1998) Nucleic Acids Research 26:697-702).

# 20 Expression of pullulanase variants

The construction of the variant of interest is accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth. This is described in detail further below.

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in the form of a protein or polypeptide, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an pullulanase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the

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vector may be an autonomously replicating vector, i.e., a as an extrachromosomal that exists entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal artificial minichromosome oran chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence that shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a pullulanase variant of the invention, especially in a bacterial host, are the promoter of the lac operon of E.coli, the Streptomyces coelicolor agarase gene dagA promoters, the promoters of the Bacillus licheniform is  $\alpha$ -amylase gene (amyL), the promoters of the Bacillus stearothermophilus maltogenic amylase gene (amyM), the promoters of the Bacillus amyloliquefaciens alpha-amylase (amyQ), the promoters of the Bacillus subtilis xylA and xylB genes, etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral alpha-amylase, A. niger acid stable alpha-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, Α. oryzae alkaline protease, A. oryzae triose phosphate isomerase (TPI) or A. nidulans acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the pullulanase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples

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of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the dal genes from B. subtilis or B. licheniformis, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise Aspergillus selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

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While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the Bacillus  $\alpha$ -amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding the pullulanase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a pullulanase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromo-

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some may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

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The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis, or Streptomyces lividans or Streptomyces murinus, or gram negative bacteria such as E.coli. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

The yeast organism may favourably be selected from a species of Saccharomyces or Schizosaccharomyces, e.g. Saccharomyces cerevisiae. The filamentous fungus may advantageously belong to a species of Aspergillus, e.g. Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of Aspergillus host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method for producing a pullulanase variant of the invention, the method comprising: cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the pullulanase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes

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(e.g., as described in catalogues of the American Type Culture Collection).

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The pullulanase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

# Testing of pullulanase

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Pullulanase variants produced by any of the methods described above may be tested, either prior to or after purification, for pullulanase activity in a screening assay which measures the ability of the variant to degrade pullulan it is desired to screen for an increased in case isoamylases activity, the ability of the variant to degrade amylopectin. The screening in step 10 in the above-mentioned random mutagenesis method of the invention may be conveniently performed by use of a filter assay based on the following procedure: A microorganism capable of expressing the mutated pullulanase of interest is incubated on a suitable medium and under suitable conditions for secretion of the enzyme, the medium being covered with two filters comprising a proteinbinding filter placed under a second filter exhibiting a low protein binding capability. The microorganism is grown on the second, top filter. Subsequent to the incubation, the bottom protein-binding filter comprising enzymes secreted from the microorganism is separated from the second filter comprising the microorganism. The protein-binding filter is then subjected to screening for the desired enzymatic activity, corresponding microbial colonies present on the second filter are identified. The first filter used for binding the enzymatic activity may be any protein-binding filter, e.g., nylon or nitrocellulose. The second filter carrying the colonies of the expression organism may be any filter that has no or low

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affinity for binding proteins, e.g., cellulose acetate or  $DURAPORA^{TM}$ .

Screening consists of treating the first filter to which the secreted protein is bound with a substrate that allows detection of the activity. The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity. The detecting compound may be immobilized by any immobilizing agent, e.g., agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents. For example, isoamylase activity can be detected by Cibacron Red labelled amylopectin, which is immobilized in agarose. isoamylase activity on this substrate produces zones on the plate with reduced red color intensity (clearing zones).

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To screen for variants with increased stability, the filter with bound pullulanase variants can be pretreated prior to the detection step described above to inactivate variants that do not have improved stability relative to the parent pullulanase. This inactivation step may consist of, but is not limited to, incubation at elevated temperatures in the presence of a buffered solution at any pH from pH 2 to 12, and/or in a buffer containing another compound known or thought to contribute to altered stability, e.g., surfactants, EDTA, EGTA, wheat flour components, or any other relevant additives. Filters so treated for a specified time are then rinsed briefly in deionized water and placed on plates for activity detection as described above. The conditions are chosen such that stabilized variants show increased enzymatic activity relative to the parent after incubation on the detection media.

To screen for variants with altered thermostability, filters with bound variants are incubated in buffer at a given pH (e.g., in the range from pH 2-12) at an elevated temperature (e.g., in the range from 50°-110°C) for a time period (e.g., from 1-20 minutes) to inactivate nearly all of the parent pullulanase, rinsed in water, then placed directly on a detection plate containing immobilized Cibacron Blue labeled pullulan and incubated until activity is detectable. As will be

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understood, thermostability and increased isoamylase activity may be tested simultaneously by using a detection plate containing immobilized Cibacron Red labeled amylopectin and incubate until activity is detectable. Moreover, pH dependent stability can be screened for by adjusting the pH of the buffer in the above inactivation step such that the parent pullulanase is inactivated, thereby allowing detection of only those variants with increased stability at the pH in question. To screen for variants with increased calcium-dependent stability, calcium chelators, such as ethylene glycol-bis(beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), is added to the inactivation buffer at a concentration such that the parent pullulanase is inactivated under conditions further defined, such as buffer pH, temperature or a specified length of incubation.

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The variants of the invention may be suitably tested by assaying the pullulan- or amylopectin-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying pullulan- and/or amylopectin-degrading host cells as described above. Further testing in regard to altered properties, including specific activity, substrate specificity, cleavage pattern, thermoactivation, thermostability, pH dependent activity or optimum, pH dependent stability, temperature dependent activity ortransglycosylation activity, stability, and any other parameter may be performed on purified variants interest, accordance with methods known in the art as described below.

Finally the present invention relates to the used of a pullulanase variant of the invention for starch conversion, both for the liquefaction and saccharification steps, in particular for producing syrups, such as dextrose or maltose syrups. A pullulanase variant of the invention may also be used for producing sweeteners; ethanol, such as fuel, industrial ethanol, from starch or whole grains instance US patent no. 5,231,017-A or US patent no. 5,756,714-A hereby incorporated by reference). Further, a pullulanase variant of the invention may also be used as cleaning

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ingredient, in laundry detergent compositions, dishwashing detergent, and hard surface cleaning compositions (see e.g., WO 99/23211, WO 97/07202 or WO 96/238874 for details on examples on cleaning compositions ingredients, the references hereby being incorporated by reference). Normally a cleaning or detergent composition also comprises at least a protease, in particular Bacillus proteases, and also one or more of the following activities: alpha-amylase, lipase, cellulase, mannanase, CGTase, maltogenic amylase.

The invention is further illustrated with reference to the following examples that are not intended to be in any way limiting to the scope of the invention as claimed.

# Determination of pullulanase activity

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Endo-pullulanase activity in NPUN is measured relative to a Novozymes pullulanase standard. One pullulanase unit (NPUN) is defined as the amount of enzyme that releases 1 micro mol glucose per minute under the standard conditions (0.7% red pullulan (Megazyme), pH 5, 40°C, 20 minutes). The activity is measured in NPUN/ml using red pullulan.

1 ml diluted sample or standard is incubated at 40°C for 2 minutes. 0.5 ml 2% red pullulan, 0.5 M KCl, 50 mM citric acid, pH 5 are added and mixed. The tubes are incubated at 40°C for 20 minutes and stopped by adding 2.5 ml 80% ethanol. The tubes are left standing at room temperature for 10-60 minutes followed by centrifugation 10 minutes at 4000 rpm. OD of the supernatants is then measured at 510 nm and the activity calculated using a standard curve.

# Expression of pullulanase from Bacillus deramificans

The pullulanase from Bacillus deramificans (SEQ ID NO: 3) is expressed in B. subtilis from a plasmid denoted pCA36. This plasmid contains the complete gene encoding the pullulanase, the expression of which is directed by the promoter from Bacillus amyloliquefaciens alpha-amylase. Further, the plasmid contains the origin of replication, oriT, from plasmid pUB110 and the cat gene from plasmid pC194 conferring resistance towards chloramphenicol. PCA36 is shown in Fig. 1.

#### **EXAMPLES**

#### EXAMPLE 1

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# Construction of Bacillus deramificans D620A variant

Gene specific primer 132011 and mutagenic primer 132012 are used to amplify by PCR an approximately 410 bp DNA fragment from the pCA36 plasmid.

The 410 bp fragment is purified from an agarose gel and used as a Mega-primer together with primer 136054 in a second PCR carried out on the same template.

The resulting approximately 1110 bp fragment is digested with restriction enzymes BsiW I and Mlu I and the resulting approximately 330 bp DNA fragment is purified and ligated with the pCA36 plasmid digested with the same enzymes. Competent Bacillus subtilis SHA273 (amylase and protease low) cells are transformed with the ligation and chlorampenicol resistant transformants are checked by colony PCR.

The mutagenesis primer 132012 introduced the D620A substitution (written in bold in the primer sequence) and introduced simultaneously a Bgl I restriction site (underlined in the primer seq.), which facilitates easy pinpointing of mutants.

Finally, DNA sequencing was carried out to verify the 25 presence of the correct mutations on the plasmid.

Primer 132011:

5' cgcttcggaatcattaggattgc 3' (SEQ ID NO: 7)

Primer 132012:

5' gcttccgttttgccttaatggcgctgc 3' (SEQ ID NO: 8)

30 Primer 136054:

5' ggccaaggctctacccgaacggc 3' (SEQ ID NO: 9)

#### EXAMPLE 2

# Construction of Bacillus deramificans E649A variant

This variant constructed as described in Example 1, except that mutagenic primer 132013 is used. The mutagenesis primer 132013 introduced the E649A substitution (written in bold in the primer sequence) and a NarI restriction site

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(underlined in the primer sequence), which facilitates easy pinpointing of mutants.

Primer 132013:

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5 5' gcactttacggggcgccatggacggg 3' (SEQ ID NO: 10)

#### EXAMPLE 3

# Thermostability test of variant of the invention

The below mentioned variants were constructed in *Bacillus*10 *deramificans* pullullanase using a megaprimer approach similar to the one described above. The following primers were used in order to introduce the various amino acid changes indicated below (all primer are written directional (5'-3'):

#### deletion (1-111)

15 Nr.167 TVB400: CATTCTGCAGCGGCCGCAAACGCTTATTTAGATGCTTCAAACC (SEQ ID NO: 11)

# deletion(1-113)

Nr.168-tvb401: CATTCTGCAGCGGCCGCAGATGATCTTGGGAATACCTATAC (SEQ ID NO: 12)

#### 20 D562P

Nr.170-tvb496:CTTTGCCACGCAGATCTCTCCCTTCGATAAAATTG (SEQ ID NO: 13)

#### G292P

NR.171-tvb497:CATTCAAACGGATCCCTATCAGGCAAAG (SEQ ID NO: 14)

# 25 G794P

Nr.172-tvb498:GTTATAATGCACCCGATGCGGTCAATG (SEQ ID NO: 15)

#### D148P

Nr. 173-tvb499:CAGCAAATAAGCCCATTCCAGTGACATCTGTG (SEQ ID NO: 16)

# N119P

30 Nr. 174-tvb500:CTTATTTAGATGCATCACCCCAGGTGC (SEQ ID NO: 17)

# N400S

Nr.175-tvb567:CAACTGCGATCGCACCAAGTGGAACGAG (SEQ ID NO: 18)

N400L

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Nr.176-tvb568:GCGATCGCACCACTTGGAACGAGGC (SEQ ID NO: 19)

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N400F

Nr.177-tvb569:CTGCGATCGCACCATTTGGAACGAGGGC (SEQ ID NO: 20)

5 N446S

Nr.178-tvb570:GACTTTTCAATTGACCCTTCTTCGGGTAT (SEQ ID NO: 21)

N446L

Nr.179-tvb571:GTCCGTGACTTTTCAATTGACCCTCTTTCGGG (SEQ ID NO: 22)

N446F

Nr.180-tvb572:GTCCGTGACTTTTCAATTGACCCTTTTTCGGGTATG (SEO ID NO: 10 23)

N504S

Nr.181-tvb573:CCAAGATAGTTGGGGTTACGATCCTCGCAAC (SEQ ID NO: 24)

N504L

Nr.182-tvb574:CCAAGATCTTTGGGGTTACGATCCTCGC (SEQ ID NO: 25) 15

N504F

Nr.183-tvb575:CCCAAGATTTTTGGGGTTACGATCCTCGC (SEQ ID NO: 26)

N735S

Nr.184-tvb576:GTCACAAGTCACGATAGCTACACCCTTTGGG (SEQ ID NO: 27)

20 N735L

> Nr.185-tvb577:GTCACAAGTCACGATCTCTACACCCTTTGGGAC (SEQ ID NO: 28)

N735F

Nr.186-tvb578:GTCACAAGTCACGATTTCTACACCCTTTGGG (SEQ ID NO: 29)

25 N789S

> Nr.187-tvb579:GCAACGACAGTAGTTATAATGCCGGCGATG (SEO ID NO: 30)

N789L

Nr.188-tvb580 GCAACGACCTTAGTTATAATGCCGGCGATG (SEQ ID NO: 31)

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N789F

Nr.189-tvb581 GCAACGACTTTAGTTATAATGCCGGCGATG (SEQ ID NO: 32)

I566A

Nr.190-TVB582 GACTTCGATAAAGCGGTACCAGAATATTATTACC (SEQ ID NO: 33)

Q485H

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Nr. 191 TVB 583 GGGATTACACATGTTCATCTTATGCCTGTTTTCG (SEQ ID NO: 34)

V551I .

10 Nr. 192 TVB 584 CATTGGGGTCAACATGGATGTTATCTATAATCATACC (SEQ ID NO: 35)

S492F

Nr. 193 TVB 585 GTTTTCGCATTTAACAGTGTCGACGAAACTGATCC (SEQ ID NO: 36)

15 D444R

Nr. 194 TVB 586 GACTTTTCCATTCGCCCGAATTCGGGTATG (SEQ ID NO: 37)

D444K

Nr 195 TVB 587 CGTGACTTTTCCATTAAACCGAATTCGGGTATG (SEQ ID NO: 20 38)

The deletion of an internal fragment corresponding to amino acids 154 to amino acid 273 was done by SOE PCR (Horton et al, 1989, Gene 77: pp61-68) utilizing the following two overlap generating oligonucleotides:

- 25 1) CCCTAGAGTAACAGATGTCACTGGAATATCC (SEQ ID NO: 39)
  - 2) GGATATTCCAGTGACATCTGTTACTCTAGGGG (SEQ ID NO: 40)

Bacillus subtilis SHA273 was transformed with plasmids harbouring the various variants and fermented.

The fermentation supernatant containing pullulanase 30 variant is subjected to the stability assay (Thermostability Assay 2 (T%)) in order to determine T% values of inactivation using the assay described above.

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46 CD TYR A 118 73.088 -6.394 6.361 1.00 18.00 147 CD2 TYR A 118 73.013 -5.460 5.86 1.00 17.79 49 CE2 TYR A 118 70.931 -5.460 5.86 1.00 17.79 149 CE2 TYR A 118 70.931 -5.460 5.86 1.00 17.79 15.00 CZ TYR A 118 70.987 -4.499 6.865 1.00 17.70 17.00 17.10

2 CA PRO A 112 80.159 5.264 -8.274 1.00 42.79
2 CA PRO A 112 79.045 4.605 -7.604 1.00 41.43
3 C PRO A 112 79.445 4.605 -7.604 1.00 41.43
5 CB PRO A 112 79.446 4.605 -7.236 1.00 39.10
6 CG PRO A 112 79.446 4.164 -9.902 1.00 43.50
8 N SER A 113 79.034 1.812 -5.120 1.00 43.50
10 C SER A 113 79.034 1.812 -5.120 1.00 35.45
11 O SER A 113 76.732 1.456 -9.902 1.00 35.45
11 O SER A 113 76.732 1.456 -4.514 1.00 33.94
12 CS SER A 113 76.732 1.456 -4.514 1.00 33.94
13 CS SER A 113 76.732 1.456 -4.514 1.00 33.94
14 N VAL A 114 77.314 -0.232 -4.504 1.00 33.94
15 C VAL A 114 77.314 -0.212 -4.504 1.00 33.94
16 C VAL A 114 77.314 -0.212 -1.972 1.00 31.03
17 C SER A 115 76.732 1.456 -4.514 1.00 31.03
18 CB VAL A 114 77.314 -0.214 -3.375 1.00 31.40
19 CCS VAL A 114 77.417 -2.419 -3.553 1.00 31.40
19 CC SER A 115 76.732 -0.222 -4.504 1.00 32.16
22 CA SER A 115 76.734 -0.232 1.00 22.70
23 C SER A 115 76.734 1.00 1.149 1.00 22.70
24 CS SER A 115 76.734 1.00 1.149 1.00 22.70
25 CB SER A 115 76.734 1.00 1.149 1.00 22.70
26 CA SER A 115 76.734 1.00 1.149 1.00 22.70
27 CA SER A 115 76.734 1.00 1.149 1.00 22.70
28 CB SER A 115 76.734 1.00 1.149 1.00 22.70
29 C A SER A 115 76.734 1.00 1.149 1.00 22.70
20 CA SER A 115 76.734 1.00 1.149 1.00 22.70
20 CA SER A 115 76.734 1.00 1.149 1.00 22.70
21 CB SER A 115 76.734 1.00 1.149 1.00 22.70
22 CA SER A 115 76.734 1.257 1.00 20.20
23 CA SER A 115 76.734 1.257 1.00 20.20
24 CA SER A 115 76.734 1.257 1.00 20.20
25 CA SER A 115 76.743 1.257 1.00 20.20
26 CA SER A 117 76.00 4.213 1.00 20.20
27 CA LAA A 117 76.00 4.213 1.00 20.20
28 CA TER A 118 72.20 1.00 20.20
29 CA TER A 118 72.20 1.00 20.20
20 CA TER A 118 72.20 1.20 1.00 20.20
20 CA TER A 118 72.20 1.20 1.00 20.20
20 CA TER A 118 72.20 1.20 1.20 1.00 20.20
20 CA TER A 118 72.20 1.20 1.2

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142 CB SER A 130 75.512 4.786 -4.600 1.00 33.55
144 N MET A 131 76.225 4.581 -7.925 1.00 34.68
145 CA MET A 131 77.225 4.581 -7.925 1.00 34.68
146 C MET A 131 77.225 4.581 -7.925 1.00 34.68
146 C MET A 131 77.225 4.581 -7.925 1.00 34.78
147 O MET A 131 77.036 2.3473 -8.788 1.00 36.36
148 CB MET A 131 77.036 2.3473 -8.788 1.00 36.36
148 CB MET A 131 77.036 2.3473 -8.788 1.00 36.36
149 CG MET A 131 77.225 4.581 -7.925 1.00 36.36
140 CG MET A 131 76.296 6.126 -10.071 1.00 46.37
151 CB MET A 131 76.296 6.126 -10.071 1.00 46.37
152 CG MET A 132 76.296 6.126 -10.071 1.00 39.31
152 CG MET A 133 76.297 1.933 -11.772 1.00 39.31
153 CG MET A 133 76.487 1.933 -11.772 1.00 39.31
154 C PRO A 132 76.487 1.933 -11.772 1.00 39.31
155 CG MET A 133 70.132 -2.052 -10.341 1.00 39.31
156 CG MET A 133 70.132 -2.052 -12.321 1.00 39.31
157 CG MET A 133 77.1329 -2.070 -11.895 1.00 38.40
156 CG MET A 133 77.232 -2.052 -12.321 1.00 38.73
157 CG MET A 133 77.1329 -2.052 -12.321 1.00 38.73
158 CG MET A 133 77.1329 -2.052 -12.321 1.00 38.13
150 CG MET A 133 77.1329 -2.052 -12.321 1.00 38.73
151 CG MET A 134 69.465 -0.663 1.064 1.00 36.21
152 CG MET A 134 69.465 -0.662 1.064 1.00 38.73
153 CG MET A 134 66.777 -1.212 -16.362 1.00 38.73
171 CG THR A 134 67.747 -1.212 -16.362 1.00 38.73
172 CG THR A 134 67.747 -1.212 -16.362 1.00 38.73
173 CG LEU A 135 68.877 -6.330 -11.367 1.00 38.73
174 N LEU A 135 68.877 -6.380 1.00 30.40 40.70
175 CG LEU A 135 68.877 -6.380 1.00 38.73
176 C LEU A 135 68.877 -6.380 1.00 38.73
177 CG LEU A 135 68.877 -6.380 1.00 38.73
178 CG LEU A 135 68.877 -6.380 1.00 39.74
189 CG ALA A 136 69.985 -7.73 -12.13 1.00 40.68
180 CG ALA A 136 69.985 -7.73 -12.13 1.00 40.69
181 CG LEU A 135 68.877 -6.380 1.00 40.89
182 CG ALA A 136 69.985 -7.73 -12.13 1.00 40.89
183 CG LEU A 135 69.787 -7.73 -12.13 1.00 42.85
184 CG ALA A 136 69.787 -7.73 -17.73 1.00 42.75
185 CG ALA A 136 69.787 -7.73 -17.73 1.00 42.75
188 CG ALA A 136 69.487 -7.73 -17.73 1.00 42.77
188 CG ALA A 136 69.487 -7.73 -17.30 1.00 40.89
189 C ALA A 134 67.487 -7.73 -17

N THR A 124 70.862 -15.914 0.062 1.00 18.31
CA THR A 124 70.213 -14.666 -1.397 1.00 19.05
C THR A 124 70.918 -3.424 0.117 1.00 19.05
C THR A 124 71.042 -13.313 1.342 1.00 19.05
CGI THR A 124 68.093 -15.653 -0.273 1.00 19.05
CGI THR A 124 68.093 -15.653 -0.273 1.00 19.05
CGI THR A 124 68.093 -15.653 -0.273 1.00 19.05
N VAL A 125 71.314 -12.463 -0.717 1.00 18.66
CM VAL A 125 71.314 -12.463 -0.717 1.00 18.66
CM VAL A 125 71.345 -10.047 -0.698 1.00 21.05
CM VAL A 125 71.485 -10.031 -1.895 1.00 21.05
CM VAL A 125 71.485 -10.031 -1.895 1.00 21.05
CM VAL A 125 71.485 -10.031 -1.895 1.00 21.49
CM LEU A 125 71.485 -10.031 -1.895 1.00 21.49
CM LEU A 126 69.993 -1.649 1.00 19.24
CM LEU A 126 69.993 -1.639 1.00 19.04
CM LEU A 127 71.265 -9.688 0.116 1.00 19.04
CM LEU A 127 71.265 -9.688 0.116 1.00 19.04
CM LEU A 127 71.265 -9.688 0.116 1.00 19.04
CM LEU A 127 71.265 -9.688 0.116 1.00 19.04
CM LEU A 127 71.265 -9.688 0.116 1.00 19.04
CM LEU A 127 71.265 -9.688 0.100 1.00 10.00

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238 CGI VAL A 144 79.645 -13.759 -2.633 1.00 24.35 240 CGZ VAL A 144 78.03 -12.421 3.949 1.00 28.03 24.13 24.1 1.00 28.03 24.1 1.00 28.04 1.00 24.13 24.1 1.00 28.03 24.1 1.00 28.04 1.00 24.1 1.00 1.00 28.04 1.00 24.1 1.00 1.00 28.04 1.00 24.1 1.00 1.00 28.04 1.00 24.1 1.00 1.00 28.04 1.00 24.1 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 28.04 1.00 1.00 1.00 28.04 1.00 28.04 1.00 28

71.558 -11.918 -16.176 1.00 41.07
69.547 -12.644 -18.479 1.00 52.75
68.067 -11.851 -20.128 1.00 54.23
67.474 -13.687 -19.039 1.00 54.23
67.474 -13.687 -19.039 1.00 54.23
67.974 -13.687 -19.039 1.00 54.23
70.708 -12.14.138 -14.777 1.00 39.52
71.379 -13.240 -13.678 1.00 37.28
70.708 -12.438 -13.041 1.00 35.69
71.862 -14.138 -14.777 1.00 39.52
71.379 -13.240 -13.550 1.00 34.74
73.425 -12.724 -12.554 1.00 35.73
73.900 -10.477 -11.730 1.00 35.73
73.910 -10.477 -11.730 1.00 35.73
73.319 -9.259 -12.589 1.00 34.52
74.538 -7.393 -13.897 1.00 33.61
74.539 -12.589 1.00 33.61
77.065 -8.690 -13.546 1.00 28.60
77.065 -8.599 -12.198 1.00 29.15
77.065 -8.599 -12.198 1.00 29.15
77.067 -9.136 -19.03 3.04
77.067 -9.136 -19.00 29.16
77.067 -9.136 -19.00 29.68
77.069 -9.120 -19.10 1.00 29.16
77.069 -9.120 -10.191 1.00 29.16
77.069 -9.120 -10.00 24.79
77.069 -9.120 -10.00 24.79
77.109 -10.281 -9.620 1.00 24.79
77.109 -10.290 -10.00 24.79
79.911 -11.224 -8.596 1.00 20.00
79.911 -11.224 -8.596 1.00 30.13
81.234 -11.093 -8.596 1.00 31.64
80.192 -12.468 -6.213 1.00 26.92
81.348 -11.292 -10.365 1.00 26.92
81.718 -13.339 -4.550 1.00 27.73
81.918 -13.339 -4.550 1.00 27.73

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72

334 CB SER A 157 68.491 -15.222 -10.214 1.00 32.34
335 CG SER A 157 67.810 -15.173 -9.012 1.00 25.08
337 CA ALA A 158 69.445 -10.774 -9.423 1.00 25.08
339 C ALA A 158 69.445 -10.774 -9.423 1.00 27.24
339 C ALA A 158 67.165 -10.022 -9.985 1.00 26.35
334 C ALA A 158 67.365 -10.022 -9.985 1.00 26.35
334 C VAL A 159 66.027 -9.812 -8.736 1.00 28.82
334 C VAL A 159 66.027 -7.709 -7.226 1.00 29.03
334 C VAL A 159 66.027 -7.709 -7.226 1.00 29.04
345 CG VAL A 159 66.027 -7.709 -7.226 1.00 29.04
346 CGI VAL A 159 66.027 -7.709 -7.226 1.00 29.04
347 CGZ VAL A 159 66.027 -7.709 -7.226 1.00 29.05
335 C SER A 160 66.29 -7.709 -7.266 1.00 29.05
335 C SER A 160 67.362 -5.076 -8.139 1.00 25.09
335 C SER A 160 67.262 -5.076 -8.139 1.00 25.09
335 C SER A 160 67.262 -5.076 -8.139 1.00 25.09
335 C SER A 160 67.262 -5.076 -8.139 1.00 25.09
335 C SER A 160 67.262 -5.076 -8.139 1.00 25.09
336 C SER A 161 67.262 -5.076 -8.139 1.00 25.09
337 C SER A 161 67.262 -5.076 -8.139 1.00 25.09
338 C ALA A 161 67.262 -5.076 -8.139 1.00 25.09
339 C SER A 161 67.262 -5.076 -8.139 1.00 25.09
340 CA SAN A 162 62.257 -4.214 1.00 28.17
361 C ASN A 162 62.257 -4.214 1.00 28.17
362 CB SER A 160 62.267 -4.314 1.00 28.17
363 CB SER A 161 67.269 -5.035 -7.366 1.00 25.08
364 CA SAN A 162 62.257 -4.214 1.00 28.17
365 CB SER A 162 62.257 -4.214 1.00 28.17
367 CB SER A 162 62.257 -4.214 1.00 28.17
368 CB SER A 162 62.257 -4.214 1.00 28.37
370 C SER A 163 56.967 -1.395 -2.296 1.00 25.08
371 C SER A 162 62.257 -4.214 1.00 28.37
372 CB SER A 163 56.967 -1.397 -3.288 1.00 25.39
373 C SER A 164 57.27 -4.214 -4.643 1.00 25.38
374 C SER A 165 62.257 -4.214 1.00 28.37
375 C SER A 165 62.257 -4.214 1.00 28.37
376 C SER A 165 62.257 -4.214 1.00 28.37
377 C SER A 163 56.967 -2.328 1.00 25.09
378 C SER A 164 62.257 -4.214 1.00 25.38
379 C SER A 164 62.257 -4.214 1.00 25.38
370 C SER A 164 62.257 -4.214 1.00 25.28
371 C SER A 164 62.257 -4.214 1.00 25.09
372 C SER A 164 62.257 -4.214 1.00 25.09
373 C SER A 164 62.257 -4.214 1.00 20.00 20.00 20.00 20.00 20.00 20.00 20.00 20.

85.046 -16.957 -3.184 1.00 32.89 85.878 -19.866 -2.699 1.00 36.86 84.457 -20.388 -2.912 1.00 39.81 83.840 -21.357 -1.955 1.00 20.00 82.931 -21.618 -0.761 1.00 20.00 82.932 -21.818 -2.659 1.00 20.00 82.373 -17.792 -5.258 1.00 34.10 84.388 -16.920 -5.880 1.00 34.10 83.10 -17.721 -5.895 1.00 33.88 83.10 -17.721 -5.895 1.00 33.96 84.318 -16.397 -9.258 1.00 20.00 86.300 -15.397 -9.258 1.00 20.00 86.300 -15.397 -9.258 1.00 20.00 86.300 -15.397 -9.258 1.00 20.00 86.300 -15.397 -9.258 1.00 20.00 86.820 -14.900 -9.971 1.00 20.00 86.820 -14.900 -9.971 1.00 20.00 86.820 -14.900 -9.971 1.00 20.00 86.820 -17.596 -1.620 1.00 30.39 81.971 -17.105 -5.571 1.00 30.99 81.005 -17.596 -17.596 -1.00 30.99 81.005 -17.596 -1.620 1.00 30.99 81.005 -17.596 -17.596 -1.00 30.99 81.005 -17.596 -17.596 -1.00 30.99 81.005 -17.596 -17.596 -17.596 -1.00 30.99 81.005 -17.596 -17.596 -17.596 -17.596 -17.596 -17.597 -9.509 1.00 30.99 81.005 -17.597 -17.595 -17.595 -17.595 -17.597 -9.641 1.00 29.73 -17.506 -17.597 -9.641 1.00 30.99 81.005 -17.597 -17.595 -17.

286 O GLU A 151
288 CG GLU A 151
289 CG GLU A 151
290 OEI GLU A 151
290 OEI GLU A 151
291 OEE GLU A 151
292 N LYS A 152
293 CA LYS A 152
294 C LYS A 152
295 CG LYS A 152
295 CG LYS A 152
296 CB LYS A 152
297 CG LYS A 152
300 NZ LYS A 152
301 N LLE A 153
302 CG LYS A 153
304 C LYS A 153
305 CG LYS A 153
306 CG LYS A 153
307 CG LYS A 154
310 C LYS A 154
311 C PRO A 154
312 C PRO A 154
313 C PRO A 154
314 CG PRO A 154
315 CG VAL A 155
316 C VAL A 155
317 CA LYR A 155
326 CG THR A 156
327 CG THR A 156
326 CG THR A 156
327 CG THR A 156
328 CG THR A 156
329 CG THR A 156
321 CG THR A 156
323 CG THR A 156

#### APPENDIX 1

430 O LEUA N 172 34.338 -8.531 -7.601 1.00 23.89
431 CB LEUA N 172 34.488 -8.06 -10.492 1.00 23.90
433 CDL LEUA N 172 35.718 -10.024 1.00 23.90
434 CD2 LEUA N 172 36.701 -6.304 -10.024 1.00 22.02
435 CD LEUA N 173 35.116 -6.409 -6.114 1.00 22.02
436 CD LEUA N 173 35.116 -6.409 -6.114 1.00 22.02
437 C GINA A 173 35.116 -6.409 -6.114 1.00 23.00
438 CD GINA N 173 35.116 -6.409 -6.114 1.00 23.00
439 CD GINA N 173 35.715 -5.008 -5.022 1.00 23.45
444 CD GINA N 173 37.125 -5.008 -5.501 1.00 20.89
444 CD GINA N 173 37.116 -6.409 -6.130 1.00 20.89
445 CD GINA N 173 37.116 -6.409 1.00 27.15
446 CD GINA N 174 37.010 -6.764 -6.897 1.00 11.02
450 CD GINA N 174 32.010 -6.764 -6.897 1.00 27.15
446 CD GINA N 174 32.010 -6.764 -6.897 1.00 27.15
447 O GINA N 174 32.010 -6.762 -5.917 1.00 27.15
448 CD GINA N 174 32.010 -6.883 -3.980 1.00 30.17
448 CD GINA N 174 32.010 -6.883 -3.980 1.00 30.17
448 CD GINA N 174 32.010 -6.764 -6.997 1.00 27.15
450 CD GINA N 174 32.010 -6.764 -1.030 1.00 30.17
451 CD GINA N 174 32.864 -4.103 -2.011 1.00 30.17
452 CD GINA N 174 32.864 -4.103 -2.011 1.00 30.17
454 CD GINA N 174 32.864 -4.103 -2.011 1.00 30.17
455 CD GINA N 174 32.864 -4.103 -2.011 1.00 30.17
456 CD GINA N 175 33.072 -11.450 -5.205 1.00 30.17
452 CD GINA N 175 33.072 -11.450 -5.174 1.00 30.40
452 CD GINA N 175 33.072 -11.450 -5.174 1.00 30.40
453 CD GINA N 174 32.864 -1.2.667 1.00 30.81
454 CD GINA N 174 32.864 -1.2.67 1.00 30.81
455 CD GINA N 174 32.864 -1.2.67 1.00 30.81
456 CD GINA N 174 32.864 -1.2.67 1.00 30.81
457 CD GINA N 176 35.31 -1.030 1.00 30.40
468 CD GINA N 176 35.490 -11.430 -2.011 1.00 31.48
469 CD GINA N 176 35.490 -11.315 -2.771 1.00 31.48
460 CD GINA N 176 35.490 -11.315 -2.771 1.00 31.48
460 CD GINA N 176 35.490 -11.315 -2.771 1.00 31.48
461 CD GINA N 176 35.490 -11.420 -0.468 1.00 33.49
462 CD GINA N 176 35.490 -11.315 -2.771 1.00 31.49
463 CD GINA N 176 35.490 -10.420 -0.468 1.00 33.49
464 CD GINA N 176 35.490 -10.420 -0.468 1.00 33.49
471 CA ANA N 178 33.439 -6.440 -0.430 1.00 33.49
472 CD GINA N 179 33.434 -6.14

382 CA THR A 165 51.200 -3.270 -4.032 1.00 25.19
384 O THR A 165 50.176 -3.008 -5.16 1.00 24.41
385 CB THR A 165 50.366 -2.262' -2.897 1.00 26.83
386 CGI THR A 165 51.832 -2.523 -1.774 1.00 26.13
389 CG THR A 165 49.533 -2.347 1.00 22.25
390 C ALA A 166 47.323 -2.397 1.00 22.25
391 N VAL A 166 47.321 -5.703 -6.108 1.00 23.36
392 CB ALA A 166 47.321 -5.703 -6.108 1.00 22.25
393 N VAL A 167 44.339 -4.399 -6.464 1.00 24.73
394 CG VAL A 167 44.399 -4.333 -7.248 1.00 19.47
395 CG VAL A 167 44.399 -4.333 -7.248 1.00 19.46
396 CJ VAL A 167 44.399 -4.399 -6.043 1.00 19.47
397 CG LEU A 167 44.399 -4.399 -6.043 1.00 19.47
398 CG VAL A 167 44.399 -4.399 -6.049 1.00 2.0.0
399 CG VAL A 167 44.399 -4.399 -6.00 1.00 19.47
40.0 N LEU A 167 44.399 -4.399 -6.049 1.00 19.47
40.1 C LEU A 168 42.257 -5.482 -8.519 1.00 18.57
40.2 C LEU A 168 41.031 -4.525 1.00 18.77
40.2 C LEU A 168 41.031 -4.525 1.00 18.77
40.3 C LEU A 168 41.031 -4.525 1.00 18.57
40.4 CC LEU A 168 41.031 -4.525 1.00 18.57
40.5 CG LEU A 168 41.031 -4.525 1.00 18.57
40.6 CD LEU A 168 41.031 -4.525 1.00 18.57
41.0 VAL A 169 39.501 -2.936 1.00 1.00
41.1 C VAL A 169 39.501 -2.936 1.00 1.00
41.2 CC VAL A 169 39.501 -2.936 1.00 1.00
41.2 CC VAL A 169 39.501 -2.936 1.00 1.00
41.2 CC VAL A 169 39.501 -2.936 1.00 1.00
41.3 CC VAL A 169 39.501 -2.936 1.00 1.00
41.4 CC CAY A 109 36.72 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
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41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 169 39.501 -2.936 1.00 1.00
41.5 CC VAL A 1

257 CB ASP A 185 42.631 -1.481 1.276 1.00 32.78 (2.27 CB ASP A 185 40.736 1.331 0.575 1.00 49.67 2.29 CD ASP A 185 40.738 1.331 0.575 1.00 49.67 2.29 CD ASP A 185 40.738 1.058 1.205 1.00 49.67 2.29 CD ASP A 186 40.727 -3.109 0.072 1.00 29.50 2.252 CA ASP A 186 40.727 -3.109 0.072 1.00 29.50 2.252 CA ASP A 186 41.256 -0.471 1.00 30.11 1.00 30.11 1.00 29.50 0.252 CA ASP A 186 41.256 -0.477 1.00 29.50 0.29 CA ASP A 186 41.259 -4.779 -0.355 1.00 29.84 40.252 CA ASP A 186 41.259 -4.779 -0.355 1.00 29.84 2.253 CA ASP A 186 39.253 -3.271 -0.280 1.00 29.89 2.253 CA ASP A 186 39.253 -3.271 -0.280 1.00 29.89 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.89 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.757 0.647 1.00 29.40 2.254 CA ASP A 187 42.390 -4.714 -0.035 1.00 29.40 2.254 CA ASP A 187 42.390 -4.714 -0.035 1.00 29.40 2.254 CA ASP A 187 42.390 -4.414 -0.035 1.00 29.40 2.254 CA ASP A 188 41.034 -4.390 0.101 1.00 29.40 2.255 CA ASP A 188 39.247 -8.562 -5.006 1.075 1.00 29.40 2.255 CA ASP A 188 39.247 -8.562 0.001 1.00 35.25 CA ASP A 188 39.247 -8.562 0.001 1.00 35.25 CA ASP A 189 41.749 -8.029 1.420 1.00 25.40

4478 O ALA A 179 31.466 -3.697 0.192 1.00 30.86 449 CB ALA A 179 33.856 -3.733 2.401 1.00 34.25 4481 CB ALA A 180 33.485 -3.733 2.401 1.00 34.25 4481 CB ALA A 180 33.485 -3.733 2.401 1.00 24.25 4484 CB ALA A 180 32.957 -2.291 -2.939 1.00 22.93 484 CB ASN A 180 32.957 -2.2621 -1.148 1.00 28.98 485 CG ASN A 180 32.957 -0.521 -1.148 1.00 29.90 485 CG ASN A 180 32.957 -0.521 -1.148 1.00 29.90 485 CG ASN A 180 32.957 -0.521 -1.148 1.00 23.94 499 CA ASN A 181 32.925 -0.522 0.100 1.00 34.34 490 C ASN A 181 32.925 -0.522 0.100 1.00 34.74 491 C ASN A 181 32.225 -1.832 -4.017 1.00 25.75 499 CA ASN A 181 32.225 -1.832 -4.017 1.00 24.74 491 C ASN A 181 32.225 -1.832 -4.017 1.00 24.74 491 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 23.94 490 C ASN A 181 32.221 -1.881 -0.599 1.00 22.19 494 400 ASN A 181 32.221 -1.881 -0.599 1.00 22.19 494 400 ASN A 182 33.60 C 0.334 -0.696 0.337 -0.599 1.00 22.19 494 400 ASN A 182 33.60 C 0.334 1.00 22.19 494 400 ASN A 182 33.60 C 0.334 1.00 20.33 4.966 0.888 A 183 33.60 C 0.238 -0.662 -9.556 1.00 20.33 4.966 0.888 A 183 33.60 C 0.238 -0.699 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.889 0.206 1.00 20.33 4.966 0.208 0.208 0.206 1.00 20.33 4.966 0.209 0.206 1.00 20.33 4.966 0.209 0.206 1.00 20.33 4.966 0.209 0.206 1.00 20.33 4.966 0.209 0.206 1.00 20.33 4.966 0.209 0.206 0.2

75

622 CA ASN A 197 58.152 -9.868 -11.964 1.00 27.61 623 C ASN A 197 56.868 -10.412 -12.426 1.00 24.74 6524 C ASN A 197 56.868 -10.412 -12.426 1.00 24.74 6525 CB ASN A 197 59.156 -9.868 -13.141 1.00 24.74 6525 CB ASN A 197 59.156 -9.868 -13.141 1.00 24.75 622 ND ASN A 197 59.143 -8.694 -15.374 1.00 22.51 629 N LEU A 198 54.09 -11.567 1.00 22.51 1.00 22.51 631 CB LEU A 198 54.09 -11.567 1.00 22.51 1.00 22.51 632 CD LEU A 198 54.097 -11.527 1.00 22.75 632 CD LEU A 198 54.037 -12.60 1.00 24.75 632 CD LEU A 198 54.037 -12.60 1.00 24.75 633 CD LEU A 198 54.037 -12.53 -9.805 1.00 24.75 633 CD LEU A 198 54.037 -12.53 -9.805 1.00 24.75 644 CD LEU A 199 55.653 -10.803 -10.724 1.00 22.56 633 CD LEU A 199 55.653 -10.803 -10.724 1.00 22.56 644 CD LEU A 199 55.653 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.653 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.654 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.654 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.654 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.654 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.655 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.655 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.655 -10.803 -10.325 1.00 23.14 644 CD LEU A 199 55.655 -10.803 -10.325 1.00 23.14 65.00 CD LEU A 200 49.591 -10.325 1.00 23.14 65.00 CD LEU A 200 49.591 -10.325 1.00 23.14 65.00 CD LEU A 200 49.591 -10.10 25.14 65.00 CD LEU A 200 49.591 -12.565 1.00 23.14 65.00 CD LEU A 200 49.326 -12.935 1.00 23.14 65.00 CD LEU A 200 49.326 -12.335 1.00 23.14 65.00 CD LEU A 201 44.460 -14.011 1.00 24.31 65.00 CD LEU A 201 44.460 -14.011 1.00 24.31 65.00 CD LEU A 201 44.80 -13.63 -12.53 1.00 23.14 65.00 CD LEU A 201 44.80 -13.63 -12.53 1.00 23.14 65.00 CD LEU A 201 44.80 -13.63 -12.53 1.00 23.14 65.00 CD LEU A 201 44.80 -13.63 -12.53 1.00 23.14 65.00 CD LEU A 201 43.421 -13.25 -13.65 1.00 23.10 65.00 CD LEU A 201 43.421 -13.25 -13.65 1.00 23.10 65.00 CD LEU A 201 43.431 -13.25 -13.65 1.00 23.10 65.00 CD LEU A 201 43.431 -13.25 -13.65 1.00 23.10 65.00 CD LEU A 201 43.431 -13.25 -13.65 1.0

718 CB TYR A 210 32.051 -14.285 1.00 25.17 712 CG TYR A 210 32.043 -1.3.064 1.00 25.77 712 CG TYR A 210 31.078 -9.699 -14.159 1.00 26.57 71 72 CE TYR A 210 31.079 -9.699 -14.159 1.00 26.59 722 CE TYR A 210 31.079 -10.066 -13.30 1.00 25.94 722 CE TYR A 210 30.647 -9.141 11.755 1.00 26.59 724 CZ TYR A 210 30.647 -9.141 11.755 1.00 26.59 724 CZ TYR A 210 30.647 -9.141 11.755 1.00 26.59 725 OH TYR A 210 30.508 -10.392 -12.330 1.00 27.30 725 OH TYR A 210 30.508 -10.392 -12.330 1.00 27.30 725 OH TYR A 211 34.770 -3.769 -15.611 1.00 21.51 725 C GIN A 211 34.770 -3.769 -15.611 1.00 21.51 725 C GIN A 211 34.770 -3.769 -15.611 1.00 21.51 725 C GIN A 211 34.770 -3.769 -15.611 1.00 21.61 725 C GIN A 211 34.770 -2.358 -15.949 1.00 20.26 733 OED GIN A 211 32.944 -0.0899 -17.595 1.00 21.64 733 OED GIN A 211 33.387 0.166 -18.950 1.00 20.26 733 OED GIN A 211 32.944 -0.0899 -17.595 1.00 21.64 733 OED GIN A 211 33.387 0.166 -18.950 1.00 20.26 744 74 72 CZ TYR A 212 38.418 -2.922 -17.274 1.00 21.64 74 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44 74 75 1.00 20.44

670 CB SER A 202 43.669 -17.32 -9.057 1.00 21.57 671 065 SER A 202 43.669 -17.32 -10.101 1.00 24.80 671 NG SER A 202 39.768 -17.32 -10.101 1.00 24.80 671 NG SER A 202 39.768 -17.32 -10.101 1.00 24.80 671 NG SER A 203 39.7355 -15.159 -17.31 1.00 28.12 672 NG GLY A 203 37.325 -16.159 -17.31 1.00 28.12 672 NG THR A 204 35.097 -17.110 -8.779 1.00 26.86 66 673 C THR A 204 35.097 -17.110 -8.779 1.00 26.80 680 C THR A 204 33.105 -15.31 -7.936 1.00 26.91 680 CG THR A 204 33.105 -18.327 -7.313 1.00 28.36 681 CG THR A 204 33.105 -18.327 -7.331 1.00 28.36 681 CG THR A 204 33.105 -18.327 -7.331 1.00 28.36 681 CG THR A 204 33.105 -18.327 -7.331 1.00 28.36 681 CG THR A 204 33.105 -18.617 -8.581 1.00 28.36 681 CG THR A 204 33.105 -18.617 -8.581 1.00 22.01 688 C CG THR A 204 33.105 -18.617 -8.581 1.00 22.01 688 C CG THR A 205 33.303 -12.22 -12.324 1.00 27.34 688 C CG THR A 205 33.303 -12.826 1.00 30.93 683 C DEU A 205 33.303 -12.829 1.00 30.93 683 C DEU A 205 33.303 -13.86 1.00 27.34 1.00 27.36 680 C DEU A 205 33.303 -13.86 1.00 27.34 1.00 27.36 680 C DEU A 205 33.303 -13.82 1.00 31.93 693 C DEU A 205 33.303 -13.82 1.00 31.93 693 C DEU A 205 33.303 -13.82 1.00 31.93 693 C DEU A 205 33.303 -13.82 1.00 31.93 693 C DEU A 205 33.303 -13.82 1.00 31.303 -13.83 1.003 1.00 31.33 1.00 31.

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815 N ASN A 221 46.107 7.509 -6.557 1.00 26.52 815 N ASN A 221 46.107 7.509 -7.535 1.00 29.39 817 C ASN A 221 47.613 7.441 -9.528 1.00 29.39 818 C ASN A 221 47.613 7.441 -9.528 1.00 29.39 818 C ASN A 221 47.613 7.441 -9.528 1.00 29.39 818 C ASN A 221 40.207 9.429 -6.270 1.00 32.29 818 C ASN A 221 48.207 9.429 -6.270 1.00 32.29 818 C ASN A 221 48.207 9.429 -6.270 1.00 32.29 818 C ASN A 221 48.207 9.429 -6.270 1.00 32.20 824 C ASN A 221 49.864 6.135 -9.421 1.00 22.27 823 ND2 ASN A 222 47.514 9.135 -9.421 1.00 28.62 825 C THR A 222 47.507 9.135 -9.421 1.00 22.27 825 C THR A 222 47.507 9.103 -9.731 1.00 22.27 825 C THR A 222 47.507 9.103 -9.731 1.00 22.27 823 C THR A 222 47.507 9.103 -9.731 1.00 22.27 823 C THR A 222 47.507 9.103 -9.731 1.00 22.27 823 C THR A 222 47.507 9.103 -9.731 1.00 22.27 823 C THR A 222 47.507 9.103 -9.731 1.00 22.27 823 C THR A 222 47.507 9.103 -9.731 1.00 22.27 833 C SER A 223 44.731 2.103 1.00 22.31 833 C SER A 223 44.731 2.103 1.00 22.31 833 C SER A 223 44.731 2.10 2.10 39.1 1.00 22.31 833 C SER A 223 44.731 0.00 22.41 84.528 C THR A 224 44.678 1.107 1.1922 1.100 22.41 84.528 C THR A 224 44.678 1.107 1.1922 1.00 22.41 84.6 1.

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862 O ASN A 227 41.160 0.489 18.779 1.00 21.15
863 CB ASN A 227 40.245 3.892 -21.307 1.00 25.98
866 CB ASN A 227 40.486 4.994 -21.332 1.00 31.48
866 ND2 ASN A 228 39.094 1.377 18.571 1.00 21.22
868 CA ASN A 228 39.094 1.377 18.571 1.00 21.22
868 CA ASN A 228 39.094 1.377 18.571 1.00 21.62
869 C ASN A 228 38.683 -1.049 -1.077 17.841 1.00 22.62
870 C ASN A 228 38.683 -0.197 17.841 1.00 22.62
871 CB ASN A 228 38.683 -0.197 17.841 1.00 22.62
872 CG ASN A 228 38.683 -0.197 17.841 1.00 21.51
873 CG ASN A 228 38.683 -0.197 17.841 1.00 21.63
874 ND2 ASN A 228 38.683 -0.197 17.841 1.00 21.63
875 C ASN A 228 38.683 -0.197 17.841 1.00 21.63
877 C VAL A 229 37.100 0.415 17.748 11.00 21.63
878 C VAL A 229 37.900 -4.412 18.687 1.00 18.06
878 C VAL A 229 37.078 -4.125 18.851 1.00 20.84
879 C VAL A 229 37.078 -4.125 18.851 1.00 20.87
888 C SER A 230 35.895 -1.769 -10.731 18.89
888 C SER A 230 35.895 -7.269 -1.071 11.00 21.95
889 C LEU A 231 35.049 -7.324 -1.9861 1.00 21.95
889 C LEU A 231 35.049 -7.324 -1.9861 1.00 21.95
889 C LEU A 231 35.049 -7.324 -1.09 31.00 21.95
889 C LEU A 231 35.049 -7.324 10.00 21.95
889 C LEU A 231 35.049 -7.324 10.00 23.94
890 C LEU A 231 35.049 -7.324 10.00 23.94
891 CD LEU A 231 35.049 -7.324 10.00 23.94
892 CD LEU A 231 35.049 -7.324 10.00 23.94
893 CD LEU A 231 35.049 -7.324 10.00 23.94
894 C SER A 230 31.548 -7.324 10.00 23.94
895 CD LEU A 231 35.049 -7.324 10.00 23.94
896 CD LEU A 231 35.049 -7.324 10.00 23.94
897 CA THR A 232 31.041 -10.449 -16.821 1.00 33.64
898 C THR A 232 31.041 -10.429 -10.03 10.00 32.67
899 C THR A 232 31.041 -10.429 -10.03 10.00 32.67
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1102 CB PRO A 258	42.684 -18.124 -22.806	1.00 27.71
1104 CG PRO A 258	42.602 -17.592 -31.815	1.00 25.18
1105 N ASN A 259	46.254 -17.532 -31.815	1.00 25.18
1106 CA ASN A 259	46.254 -17.532 -31.821	1.00 25.18
1107 CA ASN A 259	47.623 -17.532 -31.821	1.00 25.18
1108 CA ASN A 259	48.347 -17.710 -20.213	1.00 25.18
1109 CA ASN A 259	48.341 -17.787 -33.316	1.00 25.42
1101 CC ASN A 259	48.341 -17.787 -33.316	1.00 25.42
1101 CC ASN A 259	48.341 -17.787 -33.316	1.00 25.42
1101 CC ASN A 259	48.341 -17.787 -33.316	1.00 25.42
1111 ODD ASN A 259	48.341 -17.787 -33.316	1.00 24.61
1112 CC GLIN A 260	48.760 -18.771 -19.919	1.00 24.61
1113 CC GLIN A 260	50.983 -18.888 -18.882	1.00 24.62
1114 CC GLIN A 260	47.560 -19.480 -17.781	1.00 24.61
1115 CC GLIN A 260	47.560 -19.480 -17.781	1.00 22.83
1116 CC GLIN A 260	47.560 -19.480 -17.781	1.00 22.83
1117 CC GLIN A 260	47.560 -19.480 -17.781	1.00 22.83
1118 CC GLIN A 260	47.560 -19.480 -17.781	1.00 22.18
1119 CC GLIN A 261	51.624 -18.932 -10.02 24.02	
1110 CC GLIN A 261	51.624 -18.932 -10.02 24.02	
1111 CC GLIN A 261	51.624 -18.932 -10.02 24.02	
1112 CC GLIN A 261	51.624 -18.932 -10.02 24.02	
1113 CC PHE A 262	52.625 -15.897 -18.665	1.00 24.02
1114 CC RIN A 261	51.400 -17.451 -16.991	1.00 22.18
1115 CC CLIN A 261	51.400 -17.451 -16.991	1.00 22.18
1115 CC CLIN A 261	51.400 -17.451 -16.991	1.00 22.18
1116 CC CLIN A 261	51.400 -17.451 -16.991	1.00 22.18
1117 CC PHE A 262	55.622 -15.897 -18.635	1.00 22.18
1118 CC PHE A 262	55.622 -15.897 -18.691	1.00 22.18
1119 CC PHE A 262	51.401 -17.701	1.00 22.18
1111 CC PHE A 262	51.401 -17.702	1.00 22.18
1112 CC PHE A 262	51.401 -17.702	1.00 22.18
1113 CC PHE A 262	51.401 -17.702	1.00 22.18
1114 CC PHE A 262	51.401 -17.702	1.00 22.18
1115 CC PHE A 262	51.401 -17.702	1.00 22.18
116 CC PHE		

1055 CD2 PHR A 252 49.017 -9.602 -21.089 1.00 19.15
1056 CD2 PHR A 252 48.807 -9.356 -19.076 1.00 19.02
1058 CZ PHR A 252 48.807 -9.356 -19.077 1.00 19.02
1058 CZ PHR A 252 48.807 -9.356 -19.077 1.00 19.02
1059 CZ PHR A 253 43.766 -10.083 -18.207 1.00 21.01
1060 CA ASP A 253 43.766 -10.083 -18.207 1.00 21.01
1061 C ASP A 253 43.766 -10.083 -18.207 1.00 21.01
1062 CA ASP A 253 42.766 -13.01 -19.677 1.00 21.01
1063 CB ASP A 253 42.262 -13.71 -19.582 1.00 22.01
1064 CD ASP A 253 40.559 -11.192 -17.091 1.00 22.01
1065 CD ASP A 253 40.559 -11.192 -17.091 1.00 23.01
1066 CD ASP A 253 40.559 -9.643 -18.650 1.00 23.51
1066 CD ASP A 254 40.414 -15.45 -18.370 1.00 23.51
1066 CD ASP A 254 40.414 -15.645 -18.771 1.00 23.51
1067 CD ASP A 254 40.414 -15.645 -19.077 1.00 23.54
1070 CD ASP A 254 40.414 -15.645 -19.07 1.00 23.54
1071 CB SER A 254 41.210 -15.566 -19.244 1.00 23.58
1071 CD ASP A 255 39.30 -14.071 -21.643 1.00 25.58
1071 CD ASP A 255 39.30 -14.071 -21.643 1.00 25.58
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1073 CD ASP A 256 39.30 -14.071 -21.643 1.00 25.54
1074 CD ASP A 256 39.792 -12.052 -244 1.00 24.57
1075 CD ASP A 256 39.792 -12.052 -22.744 1.00 24.57
1076 CD ASP A 256 39.072 -12.652 -22.744 1.00 24.57
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7.728 7.856 7.866 7. NAMES CON CONTRACTOR OF CONTRA -8.406 -9.703 -10.375 -12.019 -13.054 -13.054 -14.451 -15.583 -15.588 -16.248 -10.921 5.294 5.294 5.294 5.294 5.294 6. APPENDIX 1

14.582 10.874 10 115.65 116.73 116.73 117.73 11 746.80 APPENDIX 1

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11583 11583 11583 11583 11583 11584 11589 11589 11589 11600 11600 11600 11610 99674 9977  $egin{array}{c} 9.00 & 9.00$ 15534 15535 15537 15537 15537 15537 15537 15557 15557 15557 15557 15557 15557 15557 15557 15557 15557 15577 15577 15577 15577 APPENDIX 1

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0.000 ALLA PROPERO P 08200088880008880008800088800088800088800088800088  $\begin{array}{c} 7.01\\ 2.01\\ 2.02\\ 3.03\\$ CAN CESS CONTRACTOR CO APPENDIX 1

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**SUBSTITUTE SHEET (RULE 26)** 

APPENDIX 1

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2398 NEE CIM A 425 50.583 -8.779 49.384 1.00 34.92 2400 C THR A 426 50.560 -10.524 44.981 10.00 25.73 2401 C THR A 426 69.876 -10.524 44.981 10.00 25.43 2401 C THR A 426 48.139 -11.054 44.030 1.00 24.91 2402 C THR A 426 69.465 -11.587 44.673 10.00 24.91 2402 C THR A 426 50.660 -12.926 43.104 1.00 24.99 2405 CC2 THR A 426 50.660 -12.926 43.104 1.00 24.99 2405 CC2 THR A 426 50.660 -12.926 43.104 1.00 24.99 2405 CC2 THR A 426 50.660 -12.926 43.104 1.00 24.99 2405 CC2 THR A 426 50.660 -12.926 43.104 1.00 24.89 2407 CA PRO A 427 47.481 -10.239 43.955 1.00 22.44 2.407 CA PRO A 427 46.131 -11.444 42.995 1.00 12.92 44.211 CG PRO A 427 46.131 -11.444 42.995 1.00 12.42 2411 CG PRO A 427 46.131 -11.444 42.995 1.00 12.42 2412 CB PRO A 427 46.131 -11.444 42.995 1.00 12.42 2411 CG PRO A 427 46.131 -11.444 42.995 1.00 12.42 2411 CG PRO A 427 46.131 -11.818 43.196 1.00 18.67 49.145 C PRO A 427 46.131 -11.818 43.196 1.00 18.67 49.145 C PRO A 427 46.131 -11.818 43.196 1.00 18.67 49.145 C PRO A 429 43.524 12.705 42.995 1.00 12.42 2411 CG PRO A 429 43.524 12.705 42.995 1.00 17.62 242 CB PRO A 429 43.524 12.705 39.396 1.00 18.69 43.196 C PRO A 430 44.231 12.995 39.136 1.00 12.01 2.42 242 CB PRO A 430 44.232 1.00 19.69 242 CB PRO A 430 44.232 -10.139 39.136 1.00 17.02 242 CB PRO A 430 44.239 43.694 19.395 1.00 17.02 243 CB PRO A 430 44.239 43.694 19.395 1.00 17.02 243 CB PRO A 430 44.239 43.694 19.30 10.0 17.0 12.31 CB PRO A 430 44.239 43.694 19.30 10.0 17.0 12.31 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 243 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 243 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 243 CB PRO A 431 40.204 -1.00 18.20 10.0 14.33 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 243 CB PRO A 431 44.239 4.967 19.0 12.24 10.0 12.31 10.0 12.31 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 243 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 10.0 16.96 10.0 12.31 CB PRO A 431 44.239 4.967 19.0 10.0 16.96 10.0 16.96 10.0 16.90 10.0 12.31 CB PRO A 431 44.239 4.967 19.0 10.0 16.90 10.0 12.31 CB PRO A 431 44.239 4.967 10.0 12.31 CB PRO A 431 44.23

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APPENDIX 1

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2707 CB THR A 465 59.637 7.293 62.406 1.00 21.74
2708 C THR A 465 59.630 7.327 50.44 50.00 1.00 19.33
2710 C LYS A 466 59.930 1.01 10 10.0 21.73
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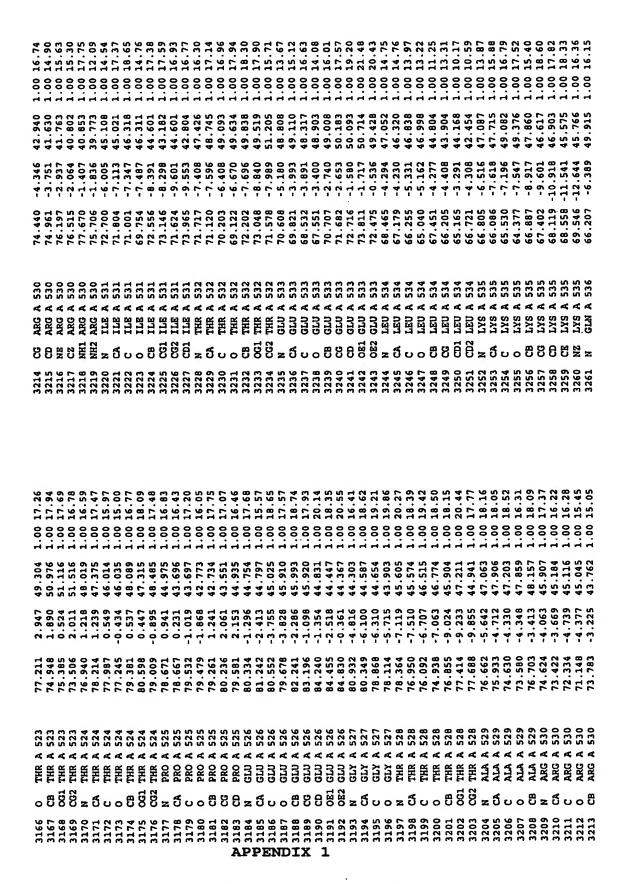
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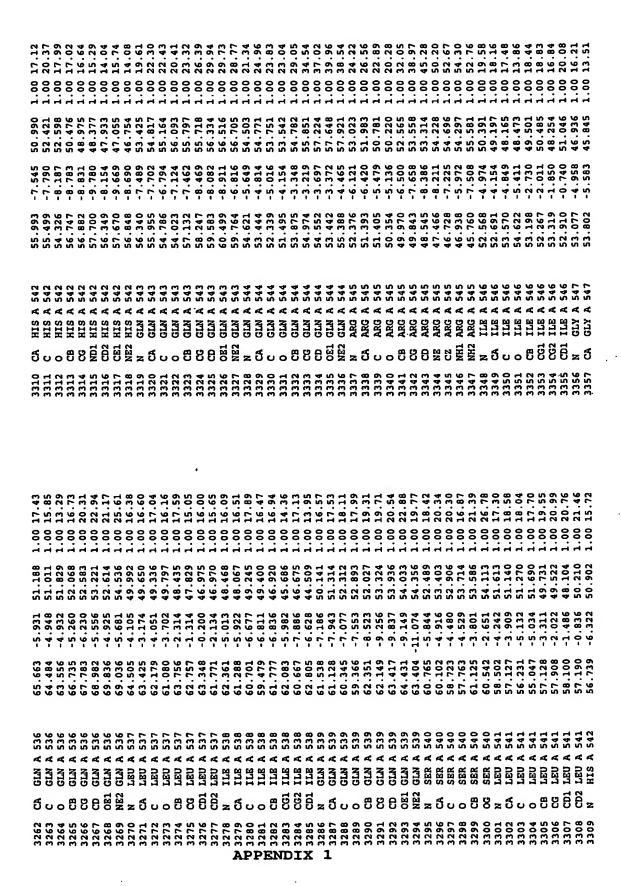
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3502 CG PHR A 565 72.397 -6.694 22.894 1.00 20.08 3504 CD PHR A 565 72.367 -7.987 23.397 1.00 26.06 3508 CEI PHR A 565 72.867 -7.987 23.397 1.00 26.06 3507 CZ PHR A 565 72.867 -7.987 23.397 1.00 26.13 3507 CZ PHR A 565 72.867 -7.987 23.397 1.00 26.43 3507 CZ PHR A 565 72.867 -7.987 23.397 1.00 26.43 3509 CZ PHR A 565 71.283 -6.128 24.577 1.00 26.43 3509 CZ PHR A 565 71.283 -7.438 25.000 1.00 22.43 3510 CZ PHR A 566 76.440 -5.285 20.148 1.00 11.00 26.83 3511 0 ASP A 566 77.10 -5.525 18.779 1.00 17.43 3511 0 ASP A 566 77.10 1-3.554 18.495 1.00 17.43 3512 CZ PASP A 566 77.201 -4.134 17.172 1.00 17.64 3513 CZ PASP A 566 77.201 -4.134 17.172 1.00 17.64 3513 CZ PASP A 566 77.201 -4.134 17.172 1.00 17.64 3513 CZ PASP A 566 77.201 -4.134 17.172 1.00 17.64 3513 CZ PASP A 566 77.201 -4.134 17.172 1.00 17.64 3513 CZ PASP A 566 75.837 -3.207 11.621 1.00 18.29 3512 CZ PASP A 566 75.837 -3.207 11.621 1.00 18.29 3512 CZ PASP A 567 79.200 -5.618 19.98 1.00 17.64 3513 CZ PASP A 567 79.200 -5.618 19.98 1.00 17.64 3513 CZ PASP A 567 79.200 -5.618 19.98 1.00 17.64 3513 CZ PASP A 567 79.200 -5.618 19.98 1.00 17.64 3513 CZ PASP A 567 79.200 -5.618 19.98 1.00 17.64 3513 CZ PASP A 567 79.200 -5.618 19.98 1.00 18.20 3522 CZ PASP A 567 79.200 -5.618 19.98 1.00 18.20 3522 CZ PASP A 567 80.648 -6.126 19.98 1.00 18.20 3522 CZ PASP A 567 80.648 -6.126 19.98 1.00 18.20 3522 CZ PASP A 567 80.648 -0.10 12.30 11.00 16.20 3522 CZ PASP A 567 80.648 -0.10 12.30 11.00 16.20 3522 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 568 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 79.549 -9.800 20.123 1.00 18.20 3523 CZ PASP A 569 77.70 8 -7.021 14.20 10.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.00 12.30 12.30 12.30 12.30 12

3454 CA ASP A 555 73.545 3.165 22.465 1.00 24.77 3455 0. ASP A 559 73.544 1.880 21.666 1.00 25.09 3455 0. ASP A 559 73.544 1.880 21.666 1.00 25.09 3455 0. ASP A 559 74.907 3.979 21.708 1.00 24.21 3455 0. D. ASP A 559 74.907 3.979 21.708 1.00 24.21 3456 0. ASP A 559 75.484 4.649 21.666 1.00 31.12 3460 0. DZ ASP A 559 76.588 5.194 21.666 1.00 31.12 3461 0. VAL A 560 74.699 0.919 19.578 1.00 24.31 3462 CA VAL A 560 74.699 0.919 19.578 1.00 24.32 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3465 CB VAL A 560 76.881 1.096 1.952 1.00 27.51 3467 CB WET A 561 76.781 -0.011 18.544 1.00 24.56 347 24.00 24.56 347 24.00 24.381 2.347 CB WET A 561 77.994 1.207 1.207 1.00 24.56 347 24.00 24.56 348 24.00 CB VAL A 562 24.56 44.00 24.56 34.00 24.56 348 24.00 CB VAL A 562 24.56 44.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.56 34.00 24.50 34.60 24.00 24.50 34.

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4. 222 2. 122 2. 123 2.071 1.730 1.501 1.501 1.501 1.501 1.501 1.501 1.501 1.501 1.271 1.115 1.115 1.271 0.050 0. 

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5597 64116 641 -0.346 -0.346 -1.602 -1.198  $\begin{array}{c} \mathbf{7} \\ \mathbf{$ ο κατο το που το APPENDIX 1

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4078 CB SER A 636 51.327 -12.920 29.022 1.00 17.93
64080 CA SER A 636 50.142 -13.62 29.76 1.00 18.54
64080 CA SER A 637 52.473 -15.869 28.956 1.00 18.54
64081 CA SER A 637 52.473 -15.869 28.956 1.00 18.54
64081 CA SER A 637 52.375 -17.314 29.219 1.00 22.54
64081 CA SER A 637 52.375 -17.314 29.219 1.00 22.04
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64081 CA SER A 637 53.454 -18.699 27.902 1.00 19.88
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64087 NDZ ASN A 637 50.142 -17.445 1.046 27.666 1.00 22.04
64088 N GLU A 638 637 50.142 -17.482 29.901 1.00 19.30
64090 CA GLU A 638 55.825 -17.482 29.901 1.00 19.63
64091 CA GLU A 638 55.825 -17.482 29.901 1.00 19.63
64092 CA GLU A 638 55.825 -18.382 29.023 1.00 22.18
64093 CA GLU A 638 55.825 -18.382 29.023 1.00 22.18
64094 CD GLU A 638 55.825 -18.382 29.023 1.00 22.18
64095 CA GLU A 638 55.825 -18.382 29.023 1.00 22.18
64097 CA GLU A 638 55.825 -18.382 29.023 1.00 22.18
64096 CA GLU A 638 55.825 -18.382 29.023 1.00 22.18
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64097 CA GLU A 639 55.428 -17.637 29.145 1.00 22.18
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4033 CB ASP A 630
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27.66 24.98 33.7.98 46.25 22.3.3.7.98 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 37.78 CANNER CONTRACTOR CONT \$2.50 APPENDIX 1

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APPENDIX 1

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4656 CB IIE A 715 31.911 -1.387 31.207 1.00 16.37
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4685 CD GIN A 719 31.00 -1.147 29.902 1.00 19.92
4686 CD GIN A 719 31.00 -1.147 29.902 1.00 19.92
4686 CD GIN A 719 31.00 -1.147 29.902

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6622 ONO WAT W 413 39.412 -12.030 33.503 1.00 27.23 6624 ONO WAT W 414 13.985 16.340 31.885 6625 ONO WAT W 415 17.995 16.340 31.885 10.00 31.89 6625 ONO WAT W 415 17.995 16.340 31.885 10.00 31.89 6625 ONO WAT W 415 17.995 16.340 31.885 10.00 31.89 6625 ONO WAT W 419 47.439 14.844 32.513 1.00 31.89 6628 ONO WAT W 420 47.439 14.844 32.513 1.00 31.89 6630 ONO WAT W 421 42.825 12.813 14.347 1.00 23.83 6631 ONO WAT W 422 65.830 14.109 14.394 16.844 32.513 10.00 31.89 6633 ONO WAT W 422 65.830 17.30 16.845 10.00 31.846 6633 ONO WAT W 425 65.830 17.30 19.00 41.00 31.846 6633 ONO WAT W 425 65.830 11.90 14.30 11.90 14.30 18.64 6633 ONO WAT W 425 65.830 11.90 14.30 11.90 14.30 18.64 6634 ONO WAT W 425 65.86 12.86 13.74 10.00 33.24 16.64 ONO WAT W 431 36.30 17.74 17.74 17.74 17.74 17.70 17.70 17.00 31.84 6635 ONO WAT W 431 434 62.37 17.74 17.70 17.70 17.00 31.80 17.70 17.64 17.70

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6718 ONO WAT W 509 40.883 -5.002 48.490 1.00 31.16 6720 ONO WAT W 510 76.430 -25.347 32.925 1.00 30.36 6721 ONO WAT W 510 71.990 1.2.764 51.11 1.00 30.30 56721 ONO WAT W 512 70.681 79.59 56.24 10.0 39.39 6722 ONO WAT W 513 20.681 0.962 54.358 1.00 39.39 6722 ONO WAT W 513 20.681 0.962 54.358 1.00 39.39 6722 ONO WAT W 515 38.997 -0.448 13.20 1.00 46.83 57.24 57.20 ONO WAT W 516 79.29 57.24 56.3 37.743 1.00 37.25 6725 ONO WAT W 516 79.29 56.24 1.00 39.39 6725 ONO WAT W 516 79.29 54.56 1.00 39.26 6725 ONO WAT W 520 79.29 64.99 5.976 1.00 39.26 6731 ONO WAT W 520 79.29 64.99 59.90 1.00 29.38 6732 ONO WAT W 520 79.29 64.99 59.90 1.00 29.38 6733 ONO WAT W 520 79.29 64.99 59.90 1.00 29.30 6733 ONO WAT W 520 79.29 64.99 59.90 1.00 29.30 6734 ONO WAT W 520 79.29 64.09 59.90 1.00 29.30 6734 ONO WAT W 520 79.30 64.30 70.20 29.30 1.00 39.20 6734 ONO WAT W 520 79.30 64.30 70.20 29.30 1.00 39.20 6734 ONO WAT W 520 79.30 64.30 70.20 29.30 1.00 39.20 6734 ONO WAT W 520 79.30 1.00 29.30 1.00 39.20 6734 ONO WAT W 520 79.30 1.00 29.30 1.00 39.20 6734 ONO WAT W 520 79.30 1.00 29.30 1.00 39.20 6734 ONO WAT W 530 79.40 1.00 29.30 1.00 39.20 6734 ONO WAT W 530 79.40 1.00 29.30 1.00 39.20 1.00 39.20 1.00 39.20 1.00 39.30 1.00 39.20 1.00

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6862 OWO WAT W 656 48.713 3.240 -19.259 1.00 36.50 6865 OWO WAT W 657 53.530 25.302 32.931 1.00 31.05 5686 OWO WAT W 659 79.662 -22.479 -0.183 1.00 50.01 6866 OWO WAT W 662 12.459 9.375 29.050 1.00 31.73 6866 OWO WAT W 661 18.141 15.650 34.72 1.00 55.01 6869 OWO WAT W 661 18.141 15.650 34.72 1.00 57.33 6869 OWO WAT W 661 18.141 15.650 34.72 1.00 50.01 6873 OWO WAT W 662 62.459 9.375 29.056 1.00 34.72 6873 OWO WAT W 664 95.356 -6.463 30.956 1.00 34.72 6873 OWO WAT W 664 95.356 -6.463 30.956 1.00 34.72 6873 OWO WAT W 664 95.356 -6.463 30.956 1.00 34.72 6873 OWO WAT W 667 70.557 14.899 27.721 1.00 47.74 6873 OWO WAT W 673 75.253 29.088 1.00 35.73 6880 OWO WAT W 673 75.26 1.00 34.72 1.00 45.76 6882 OWO WAT W 673 75.26 1.00 34.72 1.00 45.76 6882 OWO WAT W 674 674 19.27 10.42.72 1.00 45.76 6882 OWO WAT W 675 21.734 16.509 28.603 1.00 38.73 6881 OWO WAT W 682 21.734 16.509 28.603 1.00 45.66 6882 OWO WAT W 684 21.292 12.534 29.088 1.00 38.73 6881 OWO WAT W 684 21.292 12.534 29.08 1.00 45.66 6882 OWO WAT W 684 21.292 12.534 29.08 1.00 45.76 6883 OWO WAT W 686 21.202 1.00 45.67 6883 OWO WAT W 686 21.808 20.00 1.00 45.26 6883 OWO WAT W 686 21.202 1.00 31.205 1.00 45.66 6883 OWO WAT W 686 21.808 20.00 1.00 45.76 6883 OWO WAT W 689 21.808 20.00 1.00 42.76 6883 OWO WAT W 689 21.808 20.00 1.00 42.76 6893 OWO WAT W 699 21.70 41.26 69.20 0.00 WAT W 699 21.70 41.205 1.00 42.76 6893 OWO WAT W 699 21.70 41.206 1.00 42.76 6893 OWO WAT W 699 21.70 41.206 1.00 42.76 6893 OWO WAT W 699 21.70 41.206 1.00 42.76 6893 OWO WAT W 699 21.70 41.70 41.405 1.00 42.76 6893 OWO WAT W 699 21.70 41.70 41.405 1.00 42.70 6890 OWO WAT W 699 21.70 41.70 41.405 1.00 42.70 6890 OWO WAT W 699 21.70 41.70 41.405 1.00 42.70 6890 OWO WAT W 699 21.70 41.70 41.405 1.00 42.70 6890 OWO WAT W 699 21.70 41.70 41.405 1.00 42.70 6890 OWO WAT W 699 21.70 41.70 690 0.00 WAT W 696 21.70 41.70 690 0.00 WAT W 699 60.50 4.70 60.20 60.00 WAT W 690 60.50 4.70 60.20 60.00 WAT W 699 60.50 4.70 60.20 60.00 WAT W 700 60.20 60.00 WAT W 700 60.20 60.00 WAT W 700 60.20 60.00 WAT W

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7054 OWO WAT W 849 50.290 -12.920 19.561 1.00 49.79
7055 OWO WAT W 850 24.446 5.767 29.203 1.00 39.68
7056 OWO WAT W 851 44.555 9.373 55.910 1.00 30.53
7058 OWO WAT W 853 25.920 7.766 61.451 1.00 43.01
7060 OWO WAT W 854 22.922 24.189 36.031 1.00 441.20
7061 OWO WAT W 856 23.937 14.064 59.101 1.00 41.20
7062 OWO WAT W 856 23.937 14.064 59.101 1.00 46.09
7063 OWO WAT W 859 39.060 7.114 -23.052 1.00 31.07
7064 OWO WAT W 869 39.060 7.114 -23.052 1.00 31.07
7065 OWO WAT W 861 57.678 24.011 45.961 1.00 35.78
7067 OWO WAT W 862 62.731 -18.645 24.010 1.00 34.48
7067 OWO WAT W 863 55.872 23.413 49.005 1.00 52.80
7069 OWO WAT W 864 78.578 24.011 45.961 1.00 34.48
7067 OWO WAT W 865 82.130 -6.162 9.951 1.00 47.81
7069 OWO WAT W 865 82.130 -6.162 9.951 1.00 45.86
7071 OWO WAT W 865 69.803 -18.155 38.558 1.00 52.80
7072 OWO WAT W 866 69.803 -18.155 38.558 1.00 34.68

1006 ONG NAT W 801 18.046 3.941 59.147 1.00 42.238
7007 ONG NAT W 802 59.339 0.402 -2.076 1.00 52.88
7010 ONG NAT W 802 59.339 0.402 2.076 1.00 52.89
7010 ONG NAT W 803 57.132 -12.269 7.182 1.00 18.23
7011 ONG NAT W 803 57.132 -12.269 7.182 1.00 18.23
7012 ONG NAT W 803 77.944 -0.349 8.626 1.00 37.99
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7015 ONG NAT W 810 77.944 -0.349 8.628 1.00 37.99
7016 ONG NAT W 810 77.944 -0.349 8.628 1.00 37.99
7017 ONG NAT W 811 54.291 -1.443 1.1785 1.00 38.63
7018 ONG NAT W 812 51.272 1.0443 1.106 45.98
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7044 ONG NAT W 834 6.106 7.100 3.100 3.100 3.100 3.100
7044 ONG NAT W 842 7.100 3.10

**SUBSTITUTE SHEET (RULE 26)** 

## 143

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|--------------------------|-------------------|-----------------|---------------|--------------------------|------------|
| Promozyme                |                   |                 | _             | VNGNGAAYQF               |            |
| B.acidopul               |                   |                 | _             | VNGNGAAYEF               |            |
| B.deramifi               | DGNTTTIVH         | YFRPAGDIQP      | WSLWMWP       | KDGGGAEYDF               | NQPADSFGAV |
|                          | 51                |                 |               |                          | 100        |
| Promozyme                |                   | OVGLIVRKND      | WSEKNTPNDL    | HIDLAKGHEV               |            |
| B.acidopul               | · -               |                 |               | HIDLTKGHEI               |            |
| B.deramifi               |                   |                 |               | YIDLSKGNEV               |            |
|                          |                   |                 |               |                          |            |
|                          | 101               |                 |               |                          | 150        |
| Promozyme                |                   |                 |               | MPMTLADAAS               |            |
| B.acidopul               |                   |                 |               | NPMTLSDGSS               |            |
| B.deramifi               | YNEKDAEDAA        | KPAVSNAYLD      | ASNQVLVKLS    | QPLTLGEGAS               | GFTVHDDTAN |
|                          | 363               |                 |               |                          | 197        |
| Promozyme                | 151               | א אוסטידאט      | T.VCDT.OOAT.G | AANNWSPDDD               |            |
| B.acidopul               |                   | ANS             | HACDEQUADO    |                          |            |
| B.deramifi               |                   |                 |               | GS.DWAPDNH               | STLLKKVTNN |
|                          |                   |                 |               |                          |            |
|                          | 198               |                 |               |                          | 246        |
| Promozyme                | LYQLSGTLPA        | GTYQYKIALD      | HSW.NTSYPG    | NNVSLTVPQG               | GEKVTFTYIP |
| B.acidopul               | • • • • • • • • • |                 |               |                          |            |
| B.deramifi               | LYQFSGDLPE        | GNYQYKVALN      | DSWNNPSYPS    | DNINLTVPAG               | GAHVTFSYIP |
|                          |                   |                 |               | -                        | 296        |
| D                        | 247               | UDMON DDMOO     | ACTIONITY TO  | TLASAPDVTH               |            |
| Promozyme<br>B.acidopul  | SINGALDSAN        | HPNQAPPISS      | AGAGIMPAGP    | TLASAPDVSH               | TIOUGAAGVE |
| B.deramifi               |                   |                 |               | TLGEDPDVSH               |            |
| D. GCI GIIIIII           | OlimviDili.       | W. W.D.D. C. C. | ·OVALDEVIV    | 120221210                |            |
|                          | 297               | •               |               |                          | 346        |
| Promozyme                | AHNILPRNVL        | NLPRYDYSGN      | DLGNVYSKDA    | TSFRVWAPTA               | SNVQLLLYNS |
| B.acidopul               |                   |                 |               | TAFRVWAPTA               |            |
| B.deramifi               | AKQVIPRNVL        | NSSQYYYSGD      | DLGNTYTQKA    | TTFKVWAPTS               | TOVNVLLYDS |
|                          |                   |                 |               |                          |            |
|                          | 347               | ,               |               | 10/7 1/O1 MW D1O         | 396        |
| Promozyme                |                   |                 |               | YYLYQVTVNG<br>YYLYQVTVNG |            |
| B.acidopul<br>B.deramifi |                   |                 |               | YYMYEVTGQG               |            |
| B. GCI amili             | AIGSVIKIVE        | MIASGRGVNE      | AT ANGMEDIAN  | 11111111000              | Diminutin  |
|                          | 397               |                 |               |                          | 446        |
| Promozyme                |                   | MIVDLKATDP      | AGWQGDHEQT    | PANPVDEVIY               | EAHVRDFSID |
| B.acidopul               |                   |                 |               | PANPVDEVIY               |            |
| B.deramifi               | TAIAPNGTRG        | MIVDLAKTDP      | AGWNSDKHIT    | PKNIEDEVIY               | EMDVRDFSID |
|                          |                   |                 |               |                          |            |
|                          | 447               |                 |               |                          | 496        |
| Promozyme                | ANSGMKNKGK        | YLAFTEHGTK      | GPDHVKTGID    | SLKELGITTV               | QLQPVEEFNS |
| B.acidopul               |                   |                 |               | SLKELGINAV               |            |
| B.deramifi               | PNSGMKNKGK        | YLALTEKGTK      | GPDNVKTGID    | SLKQLGITHV               | QLMPVFASNS |
|                          | 497               |                 |               |                          | 546        |
| Promozyme                |                   | MCADDDMANA      | ресауаттре    | GTARITELKQ               |            |
| B.acidopul               | IDETOPNMYN        | WGYDPRNYNV      | PEGAYATTPE    | GTARITQLKQ               | LIQSIHKDRI |
| B.deramifi               | VDETDPTQDN        | WGYDPRNYDV      | PEGQYATNAN    | GNARIKEFKE               | MVLSLHREHI |
|                          |                   |                 | _             |                          |            |

|              | 547               |               |   |                          | 5.9               | 16 |
|--------------|-------------------|---------------|---|--------------------------|-------------------|----|
| Promozyme    | GVNMDVVYNH        | TEDVMVSDED    | KTVPOYYYRT                                  | DSNGNYTNGS               |                   |    |
| B.acidopul   |                   |               |   | DSAGNYTNGS               |                   |    |
| B.deramifi   |                   |               |   | DDAGNYTNGS               |                   |    |
|              |                   |               |   |                          | O I GILDZI ZI ZIZ |    |
|              | 597               |               |   |                          | 64                | 6  |
| Promozyme    | <b>PMAQKFVLDS</b> | VNYWVNEYHV    | DGFRFDLMAL                                  | LGKDTMAKIS               |                   | _  |
| B.acidopul   |                   |               |   | LGKDTMAKIS               |                   |    |
| B.deramifi   |                   |               |   | LGKDTMSKAA               |                   |    |
|              |                   |               |   | •                        |                   |    |
|              | 647               |               |   |                          | 69                | 6  |
| Promozyme    | VLYGEPWTGG        | TSGLSSDQLV    | TKGQQKGLGI                                  | GVFNDNIRNG               | LDGNVFDKTA        |    |
| B.acidopul   |                   |               |   | GVFNDNIRNG               |                   |    |
| B.deramifi   | ALYGEPWTGG        | TSALPDDQLL    | TKGAQKGMGV                                  | AVFNDNLRNA               | LDGNVFDSSA        |    |
|              |                   |               |   |                          |                   |    |
| _            | 697               |               |   |                          | 74                | 6  |
| Promozyme    |                   |               |   | ETINYVTSHD               |                   |    |
| B.acidopul   |                   |               |   | ETINYVTSHD               |                   |    |
| B.deramifi   | QGFATGATGL        | TDAIKNGVEG    | SINDFTSSPG                                  | ETINYVTSHD               | NYTLWDKIAL        |    |
|              | 747               |               |   |                          |                   | _  |
| Promozyme    |                   | TIOMET NUMBER | III DO COMO COMO COMO COMO COMO COMO COMO C |                          | 79                | 6  |
| B.acidopul   | SNESDIEADK        | INMUELAHAV    | VFTSQGVPFM                                  | QGGEEMLRTK<br>QGGEEMLRTK | GGNDNSYNAG        |    |
| B.deramifi   |                   |               |   | QGGEEMLRTK               |                   | •  |
| D. GCLAMILLI | SNPNDSEADR        | TWIDELINGAY   | VMISQGVPFM                                  | QGGEEMLRIK               | GGNDŅSYNAG        |    |
|              | 797               |               |   |                          | 84                | _  |
| Promozyme    | . • .             | KAOFKDVFDY    | FSSMTHT.RNO                                 | HPAFRMTTAD               |                   | 0  |
| B.acidopul   | DSVNOFDWSR        | KAOFENVEDY    | YSWI.THI.RDN                                | HPAFRMTTAD               | OTKONI.TELD       |    |
| B.deramifi   |                   |               |   | HPAFRMITAN               |                   |    |
|              |                   |               |   |                          | DINOIDGI III      |    |
|              | 847               |               |   |                          | · 89              | 6  |
| Promozyme    | SPTNTVAFEL        | KNYANHDTWK    | NIIVMYNPNK                                  | TSQTLNLPSG               |                   | •  |
| B.acidopul   | SPINIVAFEL        | KNHANHDKWK    | NIIVMYNPNK                                  | TAQTLTLPSG               | NWTIVGLGNO        |    |
| B.deramifi   |                   |               |   | TVATINLPSG               |                   |    |
|              |                   |               |   |                          |                   |    |
|              | 897               |               | 921   |                          |                   |    |
| Promozyme    |                   | GNVQVPAIST    |   |                          |                   |    |
| B.acidopul   | VGEKSLGHVN        | GTVEVPALST    | IILHQGTSED                                  | VIDQN                    |                   |    |
| B.deramifi   | VGESTLGQAE        | GSVQVPGISM    | MILHQEVSPD                                  | HGKK.                    |                   |    |
|              |                   |               |   |                          |                   |    |